The Regulation of Metabolic Gene Expression in Humans

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Abstract

The regulation of metabolic gene expression is fundamental to maintaining energy balance. Changes in substrate availability can alter metabolic gene expression, in order to modify the utilisation of nutrients appropriately. Metabolic gene expression can also be dysregulated in disease states. The work in this thesis examines several situations in which energy balance or metabolic substrate supply was altered, and investigates how metabolic gene expression adapted and contributed to the phenotypes observed following these interventions. All experiments in this work looked at metabolic regulation from a human perspective; with either biopsy material or cells cultured from biopsy. These experiments included;

1. The influence of postprandial fat-oxidising capacity during a calorie restricted diet of either high- or low-fat content in obese subjects.
2. Transcriptional profiling of adipose tissue in obese subjects with high- or low-postprandial fat oxidising capacity
3. High-glucose treatment of primary human myotubes (as a model of hyperglycaemia).
4. Increased PDC activation and hence carbohydrate oxidation *in vivo,* through administration of dichloroacetate.

Postprandial fat-oxidising capacity did not affect weight-loss during a calorie restricted diet, and there was no affect of diet composition. However, changes in metabolic gene expression were observed between groups over the course of the 10-week intervention. The groups which showed the greatest changes in gene expression were the low fat-oxidisers on a high fat diet and the high fat-oxidisers on a low fat diet, possibly due to a mismatch between diets and fat oxidising capacity, which required greater adaptation. Covariate analysis revealed interactions between gene expression and other phenotypic parameters. SREBP-1c showed a relationship with FFA concentrations and insulin-resistance, whilst HSL and apM1 were associated with FFA concentrations and Insulin resistance respectively, which underlines the importance of looking for underlying structures in data.

Transcriptional profiling of adipose tissue in obese subjects with high- or low-postprandial fat oxidising capacity, revealed significant differences in the expression of metabolic genes, and highlighted the importance of several transcripts; including RXRA, SREBP-1c and GLUT4 in determining the phenotype of adipose tissue. The major differences observed in gene expression between high and low fat oxidizers indicated that genes involved glucose metabolism and lipogenesis rather than beta-oxidation were the major processes that differed between the two groups. Genomic data indicated that the expression of these genes was not influenced to a major degree by polymorphisms within the population.

High-glucose treatment of primary myotubes, demonstrated the significance of ChREBP and some of its targets, in inducing the expression of lipogenic enzymes, which may be linked to the accumulation of intramyocellular triglyceride. However, these data also indicated the potential for the cell to initiate protective mechanisms, of substrate handling and lipid clearance, in response to carbohydrate oversupply. Conversely, increasing PDC activity and hence carbohydrate oxidation without altering substrate availability via infusion of dichloroacetate, did not alter the expression of metabolic genes in skeletal muscle. This reflects a capacity to deal with acute changes in the activity in metabolic genes without altering their expression.

In conclusion, the studies from this thesis show that important differences in metabolic gene expression can be observed during situations where energy balance and substrate availability are altered. However, flexibility within the metabolic networks means that acute changes can be countered without the need for induction or suppression of metabolic genes, and that during chronic alterations in nutrient supply, rapid adaptations and protective mechanisms are activated.

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Declaration

All work in this thesis was performed entirely by myself (unless otherwise stated) and in no way forms part of any other thesis. The work was carried out while I was a postgraduate student in the School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, under the supervision of Dr AJ Bennett, Dr KT Tsintzas and Professor IA Macdonald.

Paul Tisdale

List of Abbreviations

ACC	Acetyl-CoA carboxylase
Akt	Serine threonine kinase/protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
apM1	Adiponectin
AQP7	Aquaporin 7
ATGL	Adipose triglyceride lipase
bHLH/LZ	Basic helix-loop-helix-leucine zipper
BMI	Body mass index
cAMP	cyclic-adenosine monophosphate
cDNA	Complementary DNA
ChRE	Carbohydrate response element
CPT1	Carnitine palmitoyltransferase 1
Ct	Threshold cycle
DCA	Dichloroacetate
DGAT1	Diacylglycerol acyltransferase 1
ER	Endoplasmic reticulum
FAS/FASN	Fatty acid synthase
FAT/CD-36	Fatty acid translocase
FFA	Free fatty-acids
FOXO	Forkhead box
GK	Glucokinase
GLM	General linear model
GLUT4	Glucose transporter-4
HKII	Hexokinase II
HMBS	Hydroxymethylbilane synthase
HOMA-IR	Homeostasis model assessment – Insulin resistance index
HSL	Hormone sensitive lipase
IL-6	Interleukin-6
INSIG	Insulin-induced gene
IRS	Insulin receptor substrate
LDL	Low density lipoprotein
L-PK	Liver type – protein kinase
LPL	Lipoprotein lipase
LXR	Liver – X – receptor
MAPK	Mitogen activated protein kinase
MCAD	Medium-chain acyl-CoA dehydrogenase
mRNA	Messenger RNA

NUGENOB	Nutrient-gene interactions in Human obesity
P-value	Probability
pAkt	phospho-Akt
PBS	Phosphate buffer
PCA	Principle component analysis
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1	Peroxisome proliferator-activated receptor γ coactivator 1
PI3K	Phosphatidyl 3-kinase
PK	Pyruvate kinase
PKA	cAMP-dependent protein kinase
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator-activated receptor response element
PUFA	Polyunsaturated fatty acid
r	Pearson correlation
RMR	Resting metabolic rate
RPLP0	Ribosomal protein, large, P0
RXR	Retinoid X receptor
S1P	Site-1-protease
S2P	Site-2-protease
SCAP	SREBP cleavage-activating protein
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SRE	Sterol response element
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerol
TG	Triglyceride
TBS	Tris-buffered saline
Tm	melting temperature
TNFα	Tumour necrosis factor α
UCP	Uncoupling protein
VLDL	Very low density lipoprotein
WHO	World Health Organisation

Chapter 1

Introduction

1.0 Introduction

Metabolic rate in humans is tightly regulated - with the control of flux through metabolic pathways being essential as cells adapt rapidly to fluctuations in energy intake and expenditure, and also to changes in substrate availability. There are many ways in which this can be accomplished, but systems biology sets out to examine how the various components can work in concert to give both stability and adaptability in a constantly changing environment and to also to look at the pathogenesis of diseased states (Figure 1.0.3) (Kitano, 2002).

Humans are evolutionarily adapted for a 'low-energy environment', in which the availability of nutrients was limited by what could be hunted or scavenged. The advent of technology and industrialisation has changed our landscape and habitat far more rapidly than the evolutionary processes governing metabolism are capable of (Prentice *et al.*, 2008). In evolutionary terms this has been a success, since the human population has undergone exponential growth during the last few hundred years. However, in the last 40 years, metabolic diseases associated with excess calorie intake have become more prevalent (Figure 1.0.1;Figure 1.0.2) (Popkin & Doak, 1998; Kopelman, 2000).

Until the middle of the 20th century the main health issues affecting humans on a global scale were those of starvation through famine, and infectious disease. Whilst these issues are certainly still the main focus of WHO initiatives, another adversary arrived in the late 20th and early 21st century – obesity. Obesity in both developed and developing countries has grown at an unprecedented rate in recent decades. This has brought with it, a legion of associated disorders such as; type 2 diabetes mellitus, cardiovascular disease, osteoarthritis, steatohepatitis and cancer (Calle *et al.*, 2003; Kahn *et al.*, 2006; Van Gaal *et al.*, 2006; Teichtahl *et al.*, 2008). Worldwide there are, according to WHO estimates, 1.6 billion overweight and 400 million obese adults (WHO, 2006b). In the UK alone this costs the NHS £500 million, with further indirect costs to the economy of £2 billion annually (Vlad, 2003).

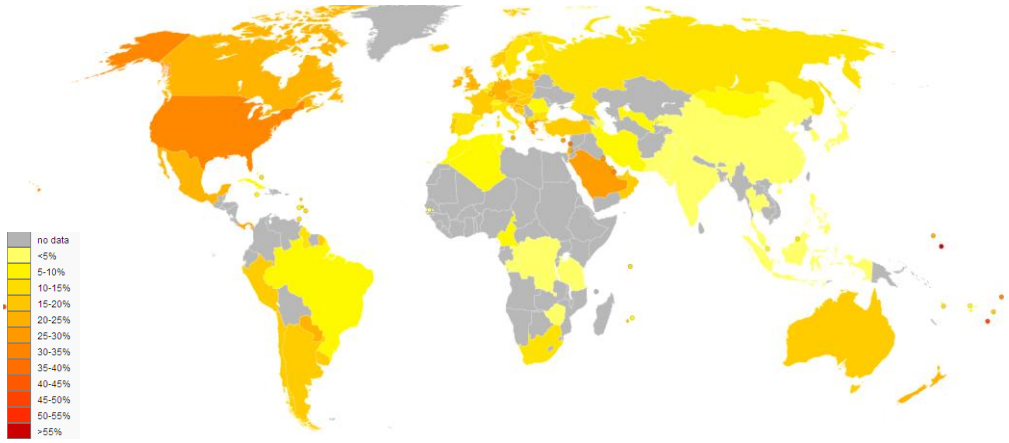


Figure 1.0.1 Global map of obesity in adult males. (% of adult population with BMI >30) per country. Data from IOTF 2008 (Lokal_Profil, 2009).

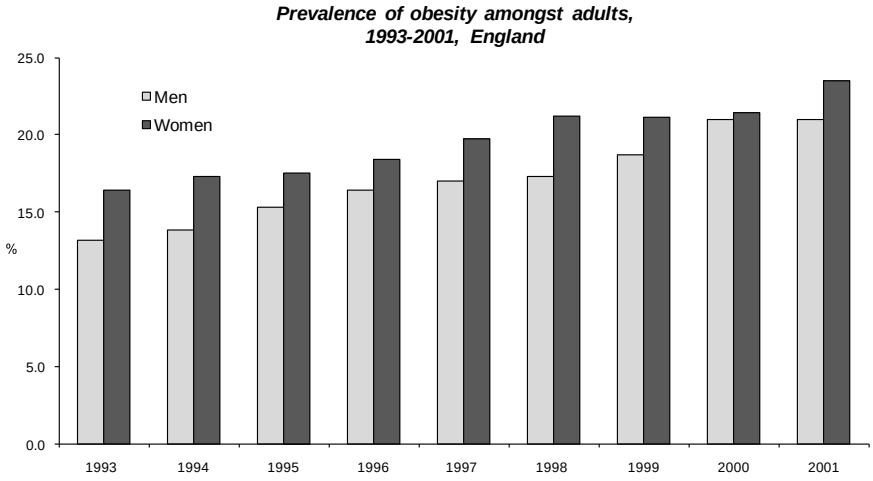


Figure 1.0.2 The increasing prevalence of obesity in England 1993-2001. Obesity in adults (16 years +), source Health Survey for England (2003). (Department of Health)

The completion of the human genome project gave rise to the possibility for scientists to understand, on a genome wide scale, how genetic differences can influence an individual's ability to regulate their metabolic rate when challenged by their environment, and also how it might affect ones propensity for disease (Jimenez-Sanchez *et al.*, 2001; Kaput & Rodriguez, 2004). There have been notable successes in linking individual genes to disease states, but these cases often apply to rare variants or small ethnic groups (Lyon & Hirschhorn, 2005). Diseases such as obesity and diabetes have a complex pathogenesis, involving many proteins, tissues and organs. It is also understood that metabolic networks are far more complicated than

simply the DNA database which comprises the genome (Janssens & van Duijn, 2008). In the short-term i.e. during high-intensity exercise, the activity of metabolic enzymes can be rapidly increased via phosphorylation and signalling cascades, which provides the required immediate response. Over the course a day it is more usual to see changes in the concentration of hormones such as insulin and glucagon, which provide changes in gene and subsequently protein expression alongside their action via phosphorylation cascades. Furthermore, it is now recognised that even prenatal dietary events in which may alter the methylation status and subsequently the level of expression of genes can affect the phenotype of offspring over generations (Ling & Groop, 2009; Tobi *et al.*, 2009). These various layers of biological interaction are all part of the metabolic response to changes in diet and energy balance.

The expression of metabolic genes is altered in response to the type of dietary substrate consumed, which affects the abundance of proteins and enzymes participating in metabolic pathways and also their activity through ligand binding or via third-party intracellular signalling events (Clarke, 1999). It is through these nutrient-gene interactions that the basis of metabolic diseases can be understood. The emerging field of nutrigenomics (studying the interaction between genes and nutrients), will aid the development of novel pharmaceutical agents for metabolic diseases, and help public health authorities in diagnosing and prescribing drugs effectively, combined with the dissemination of improved dietary advice (Muller & Kersten, 2003).

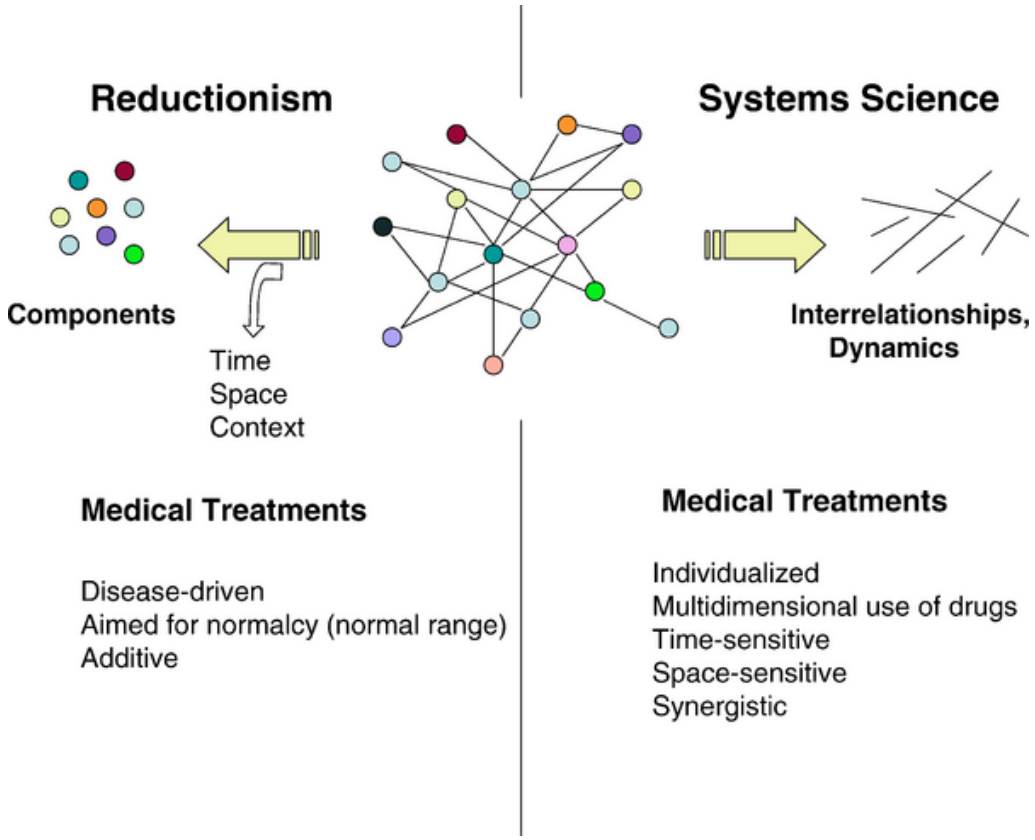


Figure 1.0.3 Different approaches to physiology. Reductionism (left) looks at the way individual components cause a particular phenotype, which can be successful in characterising monogenic disease states. Systems biology, (right) looks at the relationships between molecules, pathways and organs to understand disease progression within the entire organism, and its surrounding environment (Ahn *et al.*, 2006).

1.1 Energy balance

Energy balance is a term which describes equilibrium between energy intake and energy expenditure. During a normal 24-hour period, humans engage in various states of activity, rest and sleep, which alter energy expenditure greatly. Further to this, energy intake is confined to short periods, often 3 times per day when meals are consumed. The human body is capable of adapting to these fluctuations in energy intake and expenditure, both during the short-term and also over longer periods, such as weeks and even years. This is the concept of 'energy-balance' or metabolic homeostasis. Disturbances to energy balance can have serious health consequences, which are discussed in more detail later in this chapter.

Effective control of energy balance requires the coordination of many tissues and organs. The main peripheral tissues responsible for energy balance are muscle (chiefly skeletal muscle), the liver and adipose tissue, all of which respond to changes in substrate availability, as well as through the action of endocrine and neural stimuli. The central nervous system (CNS) is vital in integrating the response of these organs when energy balance is disturbed. The CNS largely controls energy intake (although not independently), and through both conscious physical activity and non-volitional activity such as shivering and maintaining body posture, controls energy expenditure beyond the resting-metabolic rate (RMR). Within the CNS, the hypothalamus is the site where signals from the periphery are processed, with the pituitary gland (responsible for much of the hypothalamic endocrine secretion) and arcuate nucleus being two of the most important structures in neuro-metabolic interaction. However, regions of the brain are highly interconnected, and interdependent, with other areas such as the pineal gland, which regulates circadian rhythms, and mesolimbic pathways also being involved in the control of appetite. Other endocrine tissues involved in the regulation of energy metabolism include; the thyroid gland, which regulates basal metabolic rate; the adrenal glands, from which catecholamines and corticosteroids are released, and the testis and ovaries which produce sex hormones.

The pancreas is essential for the control of blood glucose concentrations, affecting its responses through the release of insulin and glucagon – and to a lesser extent somatostatin and pancreatic polypeptide - from the islet cells. It affects glucose uptake and oxidation, but also plays a major role in regulating lipid storage and the balance between protein synthesis and breakdown.

Skeletal muscle is the site where most energy expenditure occurs. It accounts for 40-45% of body mass (Janssen *et al.*, 2000), and even at rest ~20% of energy consumption is via these tissues (Owen *et al.*, 1978; Zurlo *et al.*, 1990). It is the main site for insulin-stimulated glucose disposal and fat oxidation, but also contains stores of glycogen and intramyocellular triglycerides, which can be mobilised during exercise. Skeletal muscle is not typically considered as an endocrine organ, although it does release interleukin-6 (IL-6), which activates AMPK (Al-Khalili *et al.*, 2006; Glund *et al.*,

2007). IL-6 has also been implicated in the development of obesity (Bastard *et al.*, 2000). Adipose tissue is on the opposite side of the energy balance equation to skeletal muscle, being the main site of energy storage, particularly lipid in the form of triacylglycerol (TAG) with reserves that account for between 8 and 21% of body composition in healthy weight individuals depending on gender and activity levels. Adipose tissue is also involved in the regulation of energy balance through its function as an endocrine organ, releasing adipokines – such as adiponectin, TNFα, leptin and resistin. Leptin first came to prominence when it was its role in regulating the weight of ob/ob mice was discovered. Subsequent research has revealed that leptin acts as a sensor of nutritional status, and the secretion of leptin is proportional to fat mass. Leptin suppresses appetite, although it may be more accurate to state that reduced concentrations of circulating leptin promote appetite, since elevated leptin concentrations in obese subjects clearly do not reduce food intake. (Friedman & Halaas, 1998). Adiponectin is thought to be involved in insulin sensitization, is highly expressed in adipose tissue (Maeda *et al.*, 1996); the deletion of the adiponectin gene causes insulin resistance in mice (Kubota *et al.*, 2002; Maeda *et al.*, 2002). Unlike the majority of adipokines, adiponectin expression is reduced during obesity (Arita *et al.*, 1999). PPARγ ligands induce its expression (Maeda *et al.*, 2001), suggesting one way in which the role of PPARγ may be involved in the pathophysiology of insulin resistance. The mechanism through which adiponectin exerts its action appear to involve the suppression of hepatic glucose production (Combs *et al.*, 2001), and increasing skeletal muscle fat oxidation via activation of AMPK and PPARα (Yamauchi *et al.*, 2001; Yamauchi *et al.*, 2002).

Resistin is another adipokine which according to evidence from murine studies (Steppan *et al.*, 2001), appears to be involved in the regulation of adiposity and insulin resistance (Steppan *et al.*, 2001). These findings have not successfully been replicated in humans, therefore caution should be exercised in interpreting this role, until studies have confirmed this (Hasegawa *et al.*, 2005; Bendich *et al.*, 2010). TNFα is secreted from macrophages within adipose tissue, and is relevant to the

development of inflammation and insulin resistance, it will be discussed in more detail later in this Chapter.

The liver is a hub of substrate exchange within the periphery; it works to maintain the concentration of glucose in the bloodstream at around 5mM. The flux of metabolic pathways in the liver is closely related to the release of insulin and glucagon from the pancreas. In the postprandial state it stores carbohydrate as glycogen, and exports triglycerides for storage in adipose tissue. During the postabsorptive state this role is reverse through the breakdown of glycogen into glucose, which is then released into the blood stream. Further to this, the liver receives substrates from other tissues, such as glycerol and lactate, which it can convert back into glucose for utilisation by peripheral tissues.

The CNS can provide both behavioural adaptations and paracrine and endocrine responses to energy intake and expenditure. To do this the CNS also requires feedback from other organs, which is accomplished at many levels. Earlier in this Chapter the interaction between adipokines and satiety were described. These provide a long-term mechanism for controlling energy balance, however the gastrointestinal tract produces a range of acute responses to feeding; such as ghrelin, secreted from the stomach (Kojima *et al.*, 1999; Nakazato *et al.*, 2001; Williams & Cummings, 2005), and PYY and cholecystokinin (CCK) from the colon - all of which affect feelings of satiety through the hypothalamus (Lieverse *et al.*, 1995; Batterham *et al.*, 2002). Another acute organ-organ interaction in response to feeding is mediated by incretins. Incretins such as glucagon-like-peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) are released from the intestinal mucosa, and are part of a feed-forward mechanism by which the gut primes insulin release from the β-cells of the pancreas, before an increase in blood glucose concentrations. They also regulate the rate of gastric emptying and inhibit the release of glucagon (Holst & Gromada, 2004).

Aside from the large shifts in fuel use initiated by the endocrine system, more subtle control of fuel switching is required when substrate availability cannot meet demand or is depleted (e.g. during exercise or fasting), and at other times when there

is an excess of a particular fuel substrate. It should be stressed that these responses are usually complimentary, indeed part of endocrine responses and not necessarily distinct.

Many of the enzymes in carbohydrate and fat oxidation are prone to feedback inhibition; a notable example is the rate limiting step in glycolysis – the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate by phosphofructokinase which is inhibited by ATP (the action of which is enhanced by citrate), to self regulate flux. PFK is also activated by fructose 2,6,-bisphosphate, which in liver reduces the activity of PFK when blood glucose concentrations are low – favouring gluconeogenesis. The first step in glycolysis is mediated via hexokinase, which has a low K_m, (high affinity for glucose); however it is negatively regulated by its product G6P, which prevents the excessive use of cellular ATP to form G6P when glucose is not limiting. Pyruvate kinase (PK), which catalyses the final step in glycolysis, is positively regulated by its own substrate - phosphoenolpyruvate, and fructose 1,6-bisphosphate, along with negative regulation by ATP and acetyl-CoA (Matthews *et al.*, 1999).

The pyruvate dehydrogenase complex (PDC), catalyzes the conversion of pyruvate into acetyl-CoA, this reaction is a further example of (indirect) product inhibition. PDC activity is regulated by pyruvate dehydrogenase kinase (PDK) - which inactivates the PDC and pyruvate dehydrogenase phosphatise (PDP) which restores activity. PDK activity is enhanced by the products of the PDC, NADH and acetyl-CoA, and inhibited by its substrate pyruvate (Holness & Sugden, 2003)(Figure 1.1.1).

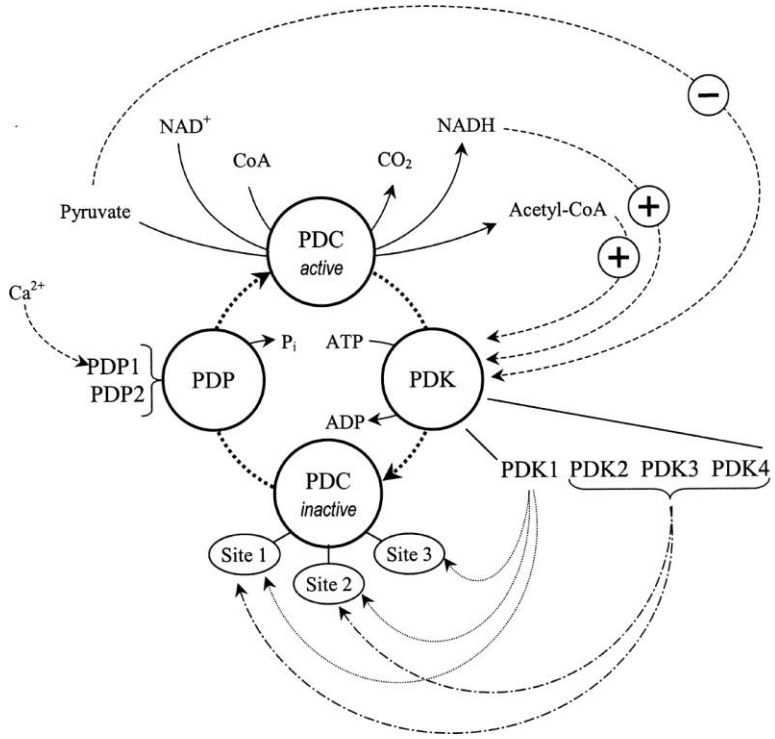


Figure 1.1.1 The regulation of PDC activity through reversible phosphorylation by PDK and PDP. PDK is activated by acetyl-CoA and NADH, which causes it to phosphorylate and inhibit PDC activity. PDC activity is restored through dephosphorylation by PDP. PDK1 is expressed at low levels in cardiac and pancreatic cells. PDK2 is expressed ubiquitously and is responsible for basal regulation of PDC activation. PDK3 is uniquely expressed in testis and the brain, whilst PDK4 is highly expressed in heart, skeletal muscle, liver, kidney and the pancreas. PDP1 is expressed in heart and at low levels in skeletal muscle, whereas PDP2 is found in liver and adipose tissue. The phosphorylation of either Site1, 2 or 3 on the E1-subunit will inactivate the PDC, however Site1 produces this action faster than the other sites, with Site3 being the slowest. All of the PDKs preferentially target Site1, but PDK2 has the highest affinity for Site1 – affecting acute changes in PDC activation, whilst PDK4 has the highest affinity for Site2. (Holness & Sugden, 2003).

During the β-oxidation of fatty acids, carnitine palmitoyltransferase-1 (CPT1) - an enzyme found in the outer mitochondrial membrane, responsible for the movement of long chain fatty acids across the membrane - forms the rate limiting step. Its significance in fuel switching lies in the interaction with carbohydrate metabolism.

The glucose fatty acid cycle first proposed by Randle in the 1960's (Randle *et al.*, 1963) is a relationship, which describes the interaction between glucose and fatty

acid oxidation. It states that FFA availability increases β-oxidation rates, which generates acetyl-CoA, and therefore increases citrate concentrations – citrate is an inhibitor of phosphofructokinase and hence reduces glycolysis/carbohydrate oxidation. Alongside this, β-oxidation also produces NADH/NAD^+, which are inhibitors of PDC activity through activation of PDKs (Figure 1.1.1). The glucose-fatty acid cycle can also be reversed; when glucose concentrations are high, the production of malonyl-CoA is increased, which inhibits CPT1 – thus reducing β-oxidation rates (Sidossis & Wolfe, 1996).

Any discussion of energy balance would be incomplete without introducing the master regulator of cellular metabolism AMP-activated protein kinase (AMPK). AMPK is an enzyme which plays a central role in maintaining energy homeostasis, therefore it merits discussion here. AMPK is formed from 3 subunits, α, β, and γ. The γ subunit is responsible for the binding of AMP, which allows AMPK to monitor fuel levels within the cell. Low levels of ATP (and therefore high levels of AMP) activate AMPK, which then targets ACC. This limits the production of malonyl-CoA, (an inhibitor of CPT1), thus increasing FFA import into the cell, along with β-oxidation. GLUT4 translocation is increased by the action of AMPK, along with upregulation of GLUT4 gene expression. AMPK plays a part role in the regulation of lipolysis in adipocytes but the mechanisms underlying these effects have yet to be fully determined (Hardie, 2008).

1.1.1 Energy balance in the postabsorptive state

The postabsorptive or fasting state is the situation commonly encountered upon waking-up in the morning. It has often been 9-11 hours since food was consumed, and as a result, plasma glucose and insulin concentrations are low, and FFA concentrations high at ~0.5mol l^{-1}. Glucagon is being released from the pancreas, and glucose is produced by glycogenolysis in the liver, with an increasing proportion from gluconeogenesis as time progresses. Glucose released from the liver is oxidised in the brain and also used by red blood cells and other cells via glycolysis. Skeletal muscle primarily oxidises FFAs due to the operation of the glucose-fatty-acid cycle,

which, via the production of acetyl-CoA indirectly inhibits glycolysis as previously described.

Net protein anabolism occurs in muscle, due to the low insulin concentrations. The amino acids released are oxidised, and the amino groups are returned to the liver as alanine, to undergo gluconeogenesis (Figure 1.1.2). In adipose tissue the low insulin concentration causes an increase in lipolysis, and the FFAs which are liberated into the bloodstream become the primary fuel substrate in skeletal muscle. FFAs are also oxidised in the liver, where the ATP produced is used for gluconeogenesis. Ketones produced as a by-product of FFA oxidation are used by the brain, muscle and adipose tissue, but do not contribute greatly as a fuel source due to the low concentration (Frayn, 2010).

1.1.2 Energy balance in the postprandial state

The postprandial state, describes the situation shortly after the consumption of a meal. After a normal meal containing a mixture of carbohydrate, fat and protein, the food passes through the stomach and into the intestine; at this point glucose absorption raises the blood glucose concentration, which causes the pancreas to secrete more insulin and less glucagon. This increases glucose uptake in muscle, adipose tissue, via the translocation of the insulin stimulated glucose transporter, GLUT4 to the plasma membrane. Glucose oxidation is rapidly increased, along with glycogen synthesis, and lipogenesis, whilst at the same time lipolysis in adipose tissue is suppressed. Glycogenolysis is inhibited in the liver by the action of insulin, and FFAs produced by *de novo* lipogenesis from glucose are transported via VLDL to adipose tissue depots.

Dietary fat passes from the ileum – where it is packaged into chylomicrons- through the lymphatic system and into the bloodstream. Lipoprotein lipase (LPL) in capillary endothelial cells liberates the FFAs and glycerol from the chylomicrons; in adipose tissue these are stored as TAG, and in skeletal muscle they are oxidised. Amino-acid adsorption from the gut, in combination with insulin signalling triggers a

switch from net-protein breakdown to net-protein synthesis in liver and muscle (Frayn, 2010).

Throughout the course of the day, each successive meal reinforces the insulin-stimulated processes, which have the effect of increasing energy storage, in the forms of glycogen and TAG. Overnight the situation slowly reverses, precipitated by a drop in plasma insulin and a rise in glucagon concentrations. As a result glycogenolysis and lipolysis take a greater role in providing the fuel sources for the body at rest.

1.1.3 High-fat diets

Excessive consumption of dietary fat has been linked to the development of obesity, hyperglycaemia, insulin resistance and atherosclerosis. Since high-fat diets are more energy-dense, it is easier to achieve a positive energy-balance when consumed over long periods. Furthermore, reduced ability to utilise increased loads of dietary fat, are associated with the obese condition (Thomas *et al.*, 1992; Astrup *et al.*, 2002). Unlike carbohydrate oxidation which is rapidly increased in response to carbohydrate feeding; fat oxidation rates fail to respond with increased dietary fat intake (Schutz *et al.*, 1989a). Flatt proposed a two-reservoir model, which represents the contribution of carbohydrate, and (the much larger) fat reserves, to whole body substrate oxidation rates. In this model the utilisation of carbohydrate is increased to a much greater extent after consumption of a meal, compared to fat oxidation, since there is a much greater potential for lipid storage than there are glycogen reserves (Flatt, 1987). Under this hypothesis, adipose depots expand with a slow increase in fat oxidation until equilibrium is restored. The type of dietary fat appears to affect the link between high fat diets and insulin resistance, with long-chain polyunsaturated omega-3 fatty acids ameliorating the potential to develop insulin resistance (Storlien *et al.*, 1987).

1.1.4 Exercise

Another situation to which the body must be able to adapt, is that of physical activity. During exercise there is a greater demand for fuel substrates, most importantly from skeletal muscle. If exercise is of high intensity for a short duration, then it is termed anaerobic. Under anaerobic conditions muscle rapidly hydrolyses ATP, which is buffered by phosphocreatine and replenished via glycogenolysis and glycolysis. This can be sustained for a short period of time, before fatigue metabolites such as lactic acid begin to interfere with muscle contraction. Longer periods of lower intensity 'aerobic' exercise utilise very different mechanisms to provide a constant source of fuel, which is derived from sources external to the contracting muscle. The somatic nervous system, and muscle contraction itself cause glycogenolysis in muscle, but it is the sympathetic nervous system and epinephrine secretion that causes lipolysis and glycogenolysis around the body. Substrates are completely oxidised during aerobic exercise, which is both more efficient in terms of ATP generation and also eliminates the build up of lactic acid in the muscle fibres (Frayn, 2010).

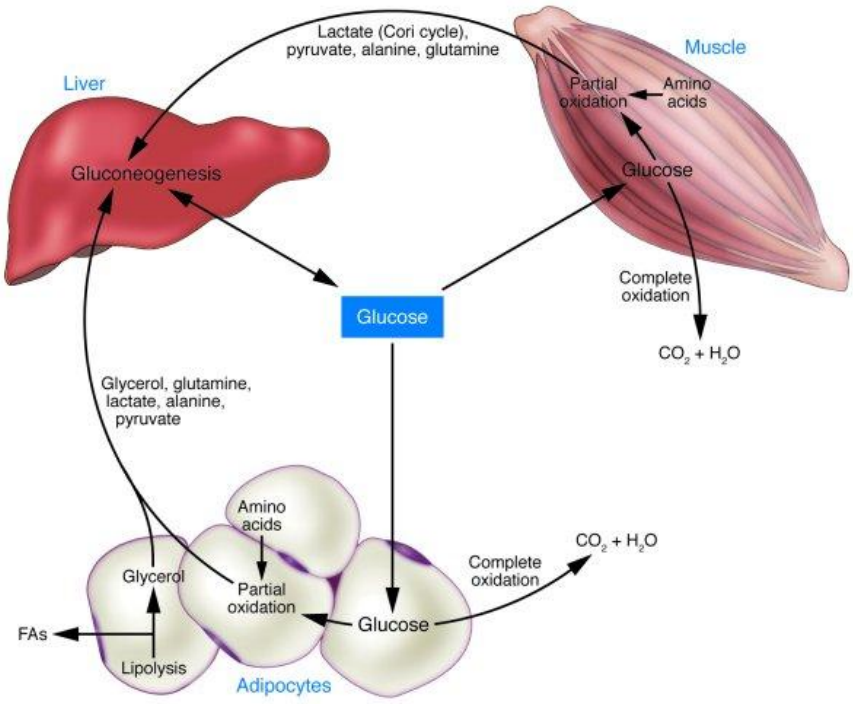


Figure 1.1.2 Glucose utilisation in peripheral tissues. Glucose is taken up by the muscle and adipose tissue where it is oxidised into CO_2 and H_2O. Depending on the state of energy balance, a variable proportion of this carbon (some derived from proteolysis) is recycled to the liver to undergo gluconeogenesis (Herman & Kahn, 2006).

1.2 Metabolic disorders

1.2.1 Obesity

Obesity is defined as a BMI >30 $kg^{-1}m^2$ (WHO, 2006b). However, there is no strict cut-off from the physiological perspective, and a sliding scale from a healthy BMI of 20, up to the morbidly obese, >35, can be observed. It is also important to recognise that BMI alone is a rather crude measurement of overweight/obesity, since it fails to take account of body composition, which in some individuals, such as athletes, is distorted due to a high BMI, but a low body fat percentage. However in the vast majority of cases an increased mass of adipose tissue accompanies the increase in body weight observed in the obese state. Obesity is a condition arising from the prolonged imbalance between energy intake and energy expenditure. Energy intake and expenditure are most often, finely balanced even over the long-term, due to the mechanisms discussed earlier in this chapter. However, situations clearly arise where this equilibrium is compromised, both in starvation and in obesity. Research has demonstrated that some groups of people are predisposed to low energy expenditure (Ravussin *et al.*, 1988), and given the obvious evolutionary benefits to conserving energy, the controversial search for a 'thrifty' gene has been given much attention. It is clear from global trends that the increasing levels of obesity require an explanation which lies outside the genome alone. Reduced levels of physical activity and excess calorie intake are obviously the main contributors to the epidemic of obesity, but since not everyone becomes obese, how these factors are co-regulated is clearly more complex.

Obesity leads to a range of secondary health implications, such as cardiovascular disease, hypertension, osteoarthritis and various cancers. However, linking BMI to increased mortality has been fraught with difficulty, due to complications arising from the fact that lower BMI is also associated with disease states, along with behavioural risks which reduced life expectancy such as smoking and alcoholism. Despite these problems, once these factors are accounted for, obesity, especially central-obesity, is clearly linked with increased mortality (Solomon & Manson, 1997).

As already discussed, obesity is linked to type 2 diabetes mellitus. However, some obese people are able to maintain a high BMI and increased adiposity (usually accompanied by hyperinsulinaemia) without developing T2DM, and are referred to as the fat-fit phenotype – fit being a relative term (Ethan, 2001). In other individuals hyperinsulinaemia can only be sustained for a period of time, before the pancreatic β-cells undergo apoptosis, leading to the development of T2DM.

Adipose tissue biology changes during the shift from lean to obese phenotypes, particularly with the onset of insulin resistance. The physical expansion of adipose tissue depots leads to changes in the vascular structure and infiltration by macrophages, this causes inflammation, and further dysregulation of adipokine production which is partially responsible progression from local to systemic insulin resistance and cardiovascular disease (Xu *et al.*, 2003; Berg & Scherer, 2005) (Figure 1.2.1). Two key adipokines linked to the development of obesity and insulin resistance are leptin and adiponectin, both of which are secreted from adipose tissue, and are often dysregulated in obesity. As previously stated, leptin is responsible for feelings of satiety. Interestingly experiments have shown that circulating leptin concentrations are very high in obese subjects, but that it fails to suppress appetite, a situation termed ‘leptin resistance’ (Enriori *et al.*, 2006), although it may be the case that leptin transport across the blood brain barrier is saturable, which limits the effects of raised leptin concentrations. Murine studies had shown some promise for treating this with exogenous leptin, however studies in humans have failed to replicate this effect (Bowles & Kopelman, 2001).

Adiponectin, on the other hand, is inversely correlated with increasing fat-mass, and since it has been observed to increase insulin sensitivity, this 'hypoadiponectinaemia' is a candidate for causing insulin resistance (Weyer *et al.*, 2001). Hypoadiponectinaemia, was partially reversed in obese women during a 2-day very low calorie diet, but restored with refeeding, which indicates that adiponectin expression responds rapidly to dietary changes, and is influenced by insulin sensitivity (Liu *et al.*, 2003). The role of ghrelin in obesity is controversial since a study in humans showed that circulating ghrelin concentrations were decreased in obese subjects – which is surprising given that lower levels would be expect to stimulate feelings of satiety, via its action in the orexigenic NPY neurons in the brain (Tschöp *et al.*, 2001). These findings, suggest that adipokines acting as satiety hormones are closely linked to the development of metabolic disease, through their ability to regulate appetite, and therefore energy intake.

The discovery of uncoupling proteins generated a great deal of interest surrounding their role in energy expenditure. This family of proteins were first discovered in brown adipose tissue, where UCP-1 has the ability to 'uncouple' ATP production from oxygen consumption by allowing protons to leak back across the inner mitochondrial membrane (Ricquier *et al.*, 1991). This has the effect of releasing energy in the form of heat as the potential energy stored in the proton gradient is dissipated. UCP-2 and -3, which are found in white adipose tissue and skeletal muscle respectively, were then isolated, but their function appears to be different in these tissues. Interestingly, there is increasing evidence that suggests UCP3 is instrumental in regulating lipid metabolism. Fasting and high-fat feeding in rodents, up-regulate expression of UCP3 mRNA (Ricquier & Bouillaud, 2000). Although these two interventions have opposite effects on energy expenditure, they both increase the concentration of circulating free fatty acids (FFA's) and consequently, increase lipid oxidation rates. More recently it has been suggested that UCP3 might facilitate lipid oxidation by transporting FFA anions out of the mitochondria, or reducing limiting the production of reactive oxygen species (Nagy *et al.*, 2004). UCP3 expression appears to be upregulated by PPARα and δ in response to increased circulating FFAs in

skeletal muscle (Villarroya *et al.*, 2007). Pima Indians are an ethnic group which have the highest known incidence of obesity and diabetes, and a lower RMR, which may translate into altered body composition over a number of years. Polymorphisms in the UCPs have been correlated with resting energy expenditure (but not obesity) in Pima Indians and Caucasians (Walder *et al.*, 1998; Schrauwen *et al.*, 1999).

Attempts to treat obesity have traditionally relied upon advice to reduce calorie intake and increase levels of physical activity. This remains the most common and effective method of treatment in the early stages of obesity. However, once obesity or T2DM are sufficiently advanced, compliance with such regimes is poor, and both secondary disorders and physical disability compound this.

Calorie restriction (CR) or 'dieting' is the concept of deliberately reducing energy intake, usually to achieve weight loss. It has been promoted for many years (alongside exercise) as the most effective way to induce weight loss without using surgery.

CR diets in obese subjects shift energy balance into negative territory, which when sustained, lead to weight loss until equilibrium is re-established and body weight is maintained at a lower mass. Humans have evolved to survive for considerable periods with reduced food intake, and the mechanisms which regulate this, also operate during weight loss diets. Once an individual begins a CR diet, leptin and thyroid hormone levels decrease which reduce energy expenditure considerably, thus requiring additional reductions in energy intake just to begin the process of weight loss – made harder by the reduced action of leptin which promotes feelings of hunger (ghrelin also providing an acute response). Once glycogen reserves have been exhausted, the adipose tissue stores will become the main fuel source. The high energy density of stored TAGs means that weight loss is a slow process – especially since physical activity is usually reduced in obese individuals. Reduced levels of physical activity precipitate a drop in the expression of peroxisome proliferator-activated receptor-γ -1-α (PGC1α) – a transcription factor which regulates genes involved in energy metabolism and mitochondrial biogenesis, this is a factor which contributes to poor oxidative capacity in skeletal muscle. Reductions in fat mass are eventually accompanied by loss of lean mass, which requires the dieter to make

further reductions to their energy intake to maintain weight loss. These difficulties outline why CR diets are in some individuals so unsuccessful. The effects of dietary composition during CR are still being examined, with long-term success rates and compliance obfuscating the issue. A large multi-centre study by Viguerie *et al* appears to show that CR is of greater importance than dietary composition, in promoting weight loss and reducing insulin resistance (Viguerie *et al.*, 2005). Very-low carbohydrate diets have become popular in recent years, but concerns exist about both the side-effects, and long term efficacy of these programmes (Astrup *et al.*, 2004).

During the last decade, the number of surgical interventions, as a treatment for obesity, has increased exponentially (Burns *et al.*, 2010). Bariatric surgery produces a dramatic and permanent reduction in energy intake, but the costs and risks of surgery must be balanced against the necessity for this operation.

Pharmacological attempts to induce weight loss have had some success, but are prone to have such adverse side effects, that treatment is either highly unpleasant, or even dangerous. At present only one drug, Orlistat, is prescribed on the NHS to promote weight loss. Orlistat, (tetrahydrolipstatin) prevents the release of pancreatic lipase, which reduces the uptake of dietary fat in the ileum. Sibutramine, which was previously prescribed, but has now been withdrawn from use, worked by increasing the concentration of serotonin (5-HT) in the brain, which reduced appetite and in-turn calorie intake. These drugs can significantly reduce weight, but only by a small percentage and therefore, other mechanisms for reducing weight by pharmacological intervention are being sought (Davidson *et al.*, 1999; Arterburn *et al.*, 2004). Rimonabant, a CB1 antagonist, was a promising pharmaceutical agent developed to target the CB1 receptor, which helps in the regulation of appetite. It was dropped from development after psychological disturbances were observed during trials, however recent research has shown that genotyping of patients could eliminate this risk (Topol *et al.*). Mutations in the melanocortin receptor (MC4R) have been linked with obesity, and therefore agonists are being actively developed by pharmaceutical companies. MC4R is a second order target for leptin, the effects of which are mediated through the inhibitory NPY/AgRP and stimulatory POMC/CART neurons in the Arcuate

nucleus. However, a range of side-effects have stifled efforts thus far (Adan *et al.*, 2006).

The progression to Type 2 Diabetes Mellitus is often associated (in 80% of cases in the UK), with overweight and obese conditions, however, despite this fact, the causes of diabetes at the molecular level are still not fully understood. When diabetes is present in the overweight/obese condition, it is often referred to as metabolic syndrome (Alberti *et al.*, 2006).

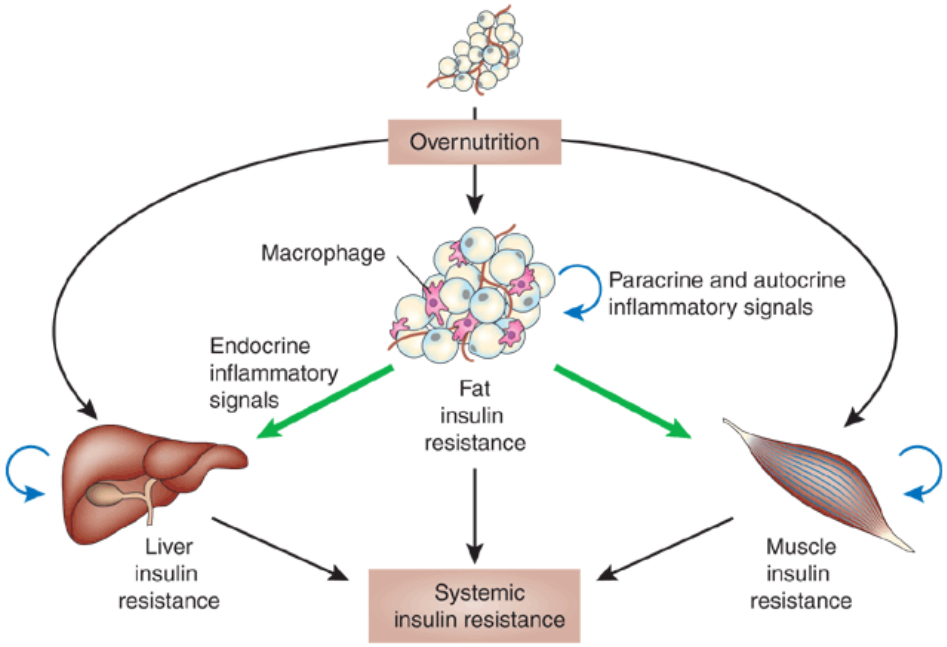


Figure 1.2.1 The link between overnutrition and insulin resistance. The development of systemic insulin resistance in response to the obese state. In obesity the expanding adipose tissue mass is infiltrated by macrophages, which scavenge for adipocytes. These macrophages release pro-inflammatory cytokines such as TNFα and IL-6. These signals lead to increased FFA release, and reduced production of adipokines which causes local insulin resistance within the adipose tissue. The release of pro-inflammatory cytokines, and concomitant disruption of adipokines, along with increased ectopic fat deposition, eventually lead to the development of systemic insulin resistance, hyperinsulinaemia and potentially T2DM (de Luca & Olefsky, 2006).

1.2.2 Diabetes

Diabetes mellitus is a metabolic disease characterised by persistently high blood glucose concentrations (≥7.0mmol/l (WHO, 2006a). High blood glucose, termed hyperglycaemia, can cause a range of secondary diseases which such as kidney failure, neuropathy, retinopathy and infection – which all lead to an increased risk of mortality.

There are two main categories of diabetes. The first, Type 1 diabetes mellitus, (also referred to as early-onset or insulin dependent diabetes mellitus) is caused by auto-immune destruction of pancreatic β-cells, which renders the patient unable to produce endogenous insulin. Successful treatment of Type 1 diabetes can achieved through the administration of regular insulin injections, and careful monitoring of blood glucose concentrations. Type 1diabetes is not highly-prevalent with only about 0.5% of the UK population being affected.

Type 2 diabetes mellitus (more rarely called; late-onset, or non-insulin dependent diabetes) has a different pathogenesis to Type 1 diabetes. Insulin secretion in T2DM is partially impaired, but the main cause is the failure of insulin action on metabolic tissues – this is known as insulin resistance. Insulin resistance in T2DM occurs even at normal insulin concentrations. Due to impaired glucose uptake in muscle and adipose tissues (principally via GLUT4) blood glucose concentrations remain high even in the fasting state. Raised levels of glucagon are also observed in diabetes, as a result of increased sympatho-adrenal activity, which promotes glycogenolysis, lipolysis, gluconeogenesis and ketogenesis, whilst inhibiting lipogenesis and glycolysis. The liver adapts to this situation by producing glucose via gluconeogenesis. If untreated this can lead to a dangerous situation termed ketoacidosis. Chronically high levels of insulin secretion, as a mechanism of combating insulin resistance, along with lipotoxicity in pancreatic β-cells causes apoptosis, and when this occurs insulin can no longer be endogenously produced, leading to a clinical diagnosis of T2DM (Unger & Zhou, 2001).

As previously stated, the development of T2DM is often attributed to obesity, although in some cases it affects lean people, especially the elderly. The

pathogenesis of T2DM in lean cases is different to that of obese subjects -. The heterogeneity of T2DM suggests that it is important to differentiate between these groups when conducting research.

The mechanisms surrounding the switch from the obese insulin resistant state to T2DM are unclear, but there has been strong implication of the role of PPARγ (Barroso *et al.*, 1999; Kubota *et al.*, 1999). Theories suggest that it could be due to the expansion of adipose tissue mass which excludes FFAs from binding with PPARγ, and therefore reducing lipid storage potential through adipocyte differentiation and further expansion. This may then lead to ectopic fat storage, through various pathways, increasing the risk of systemic insulin resistance (Balasubramanyam & Mohan, 2000). Initially insulin resistance is compensated for by increasing insulin secretion and β-cell mass, however there is a point at which this is unable to keep pace with increases in IR, and glucose toxicity as a result of chronic hyperglycaemia, combined with metabolic stress, leads to β-cell apoptosis and eventually T2DM.

Treatment for type 2 diabetes mellitus usually begins with dietary advice, suggesting a weight-loss diet in those patients who are obese; insulin injection may be used in some cases. Pharmaceutical treatment has utilises two main approaches; increasing insulin secretion and increasing insulin sensitivity.

Sulfonylureas increase insulin secretion from β-cells in the pancreas, but a balance has to be struck between the half-life of the drug and the potential for causing hypoglycaemia, furthermore, poor administration can cause weight gain through excessive release of insulin. Incretin mimetics, such as Exenatide, bind to GLP receptors in the pancreas and have a similar end result to sulfonylureas, whereby insulin secretion is increased - although this is action is initiated via a different route.

Metformin is another commonly prescribed anti-diabetic drug. It acts by suppressing endogenous glucose production (which contributes up to 20% of the raised blood glucose concentrations in T2DM) through activation of AMPK, and through increasing insulin sensitivity in peripheral tissues – which further reduces hyperglycaemia Thiazolidinediones, which also increase insulin sensitivity, have been prescribed since the 1990s, but many have been withdrawn due to adverse

cardiovascular side-effects. The two main prescription drugs in this class are rosiglitazone, and pioglitazone, which increase insulin sensitivity through binding to the PPARs, having greatest affinity for PPARγ. The agonism of PPARγ, is believed to improve lipid storage by adipose tissue, thus reducing the substrate competition in skeletal muscle. This allows increased glucose utilisation, alleviating the effects of hyperglycaemia, although it does come with the drawback of increasing adiposity. Recently there have been calls for Rosiglitazone to be withdrawn, due to some evidence that it may also increase the risk of myocardial infarction (Cohen, 2010).

1.3 Transcriptional regulation in metabolism

The regulation of gene expression is carried out in many ways, with interactions between ligands, transcription factors and the promoters of target genes. In addition to these transcriptional mechanisms; translation and post-translational modification also play an important role. In reality, the concept of pathways – which have been very successful used in the study of enzyme-metabolite-enzyme interactions – cannot be readily applied to gene expression. However, studies have consistently shown a set of transcription factors to be involved in the control of metabolic gene expression. The list below is not exhaustive, but provides and introduction to the major regulators of metabolic gene expression, particularly in the context of carbohydrate and fat oxidation.

1.3.1 Sterol Response Element Binding Protein (SREBP)

Sterol regulatory element binding proteins (SREBP) are transcription factors belonging to the class containing basic-helix-loop-helix-leucine-zipper (bHLH/LZ) domains (Kim *et al.*, 1995). To date, three variants of SREBP have been described: SREBP-1a and SREBP-1c are splice variants of the same gene (SREBF1), transcribed via alternate promoters. SREBP-2 is transcribed from a separate locus (SREBF2) and only shares 50% homology with SREBP1, but is expressed ubiquitously (Hua *et al.*, 1993). SREBP-1a is expressed in most tissues at a low level,

but at more significant levels in cell lines and the intestine. SREBP-1c is expressed at higher levels in skeletal muscle, adipose tissue, liver, the adrenal medulla and the brain (Shimomura *et al.*, 1997).

The SREBP-1 isoforms are primarily responsible for regulating fatty acid biosynthetic genes, whereas SREBP-2 controls cholesterol biosynthesis (Horton *et al.*, 2002). SREBP-1c has a slightly shorter transactivation domain than SREBP-1a, which gives it a lower affinity for the promoters of target genes (Shimano *et al.*, 1997).

The mechanism which regulates the activity of SREBPs is known as 'Regulated intramembrane proteolysis (Rip) (Brown *et al.*, 2000). In this system, SREBPs are translated from mRNA into 125 kDa precursor proteins which are then localized to the endoplasmic reticulum (ER). In the ER SREBPs associate with two proteins; SREBP-cleavage activating protein (SCAP), and insulin-induced gene (INSIG) (Nohturfft *et al.*, 1998). SREBPs are retained in the ER, and only translocate to the Golgi apparatus for activation after being released through one of two mechanisms. In the first case, if sterol levels are reduced, SCAP no longer binds to INSIG, this applies to SREBP-2 and SREBP-1a (Brown & Goldstein, 1999). In the second case a rise in insulin concentrations causes INSIG to release the SREBP-1c:SCAP complex (Hegarty *et al.*, 2005). In both situations the SREBPs are then free to translocate to the Golgi apparatus via COPII vesicles (Sun *et al.*, 2005). Once they arrive in the Golgi apparatus, they are cleaved by two proteases; Site-1 protease (S1P) and Site-2 protease (S2P), into a transcriptionally active 68 kDa proteins. The mature SREBP is then free to bind with SREs in the nucleus, where it can initiate transcription of target genes (Wang *et al.*, 1994; Sakai *et al.*, 1998) (Figure 1.3.1).

Aside from their mechanical role, INSIG proteins refine the regulation of SREBPs, through alternative splicing. Three variants of INSIG; -1, -2a and -2b are recognised (-2a and -2b are splice variants of the same gene). Both INSIG-1 and -2 are required for sterol mediated retention of SREBP:SCAP in the ER (Yabe *et al.*, 2002; Engelking *et al.*, 2004). INSIG-1 is readily degraded by the ubiquitin-proteasome pathway, but INSIG-2 has a comparatively longer half-life (Gong *et al.*, 2006). INSIG-1 uniquely requires nuclear SREBPs for its expression. Whilst INSIG-2 binds SCAP only

in the presence of sterols preventing release from the ER (Yabe *et al.*, 2002). In liver, INSIG-1 is induced by insulin, but INSIG-2a (the more prevalent hepatic isotype of INSIG-2) is suppressed (Yabe *et al.*, 2003). More recent experiments show that SREBP-1c:SCAP associates selectively with INSIG-2a, and that insulin promotes degradation of INSIG-2a, which enhances the export of SREBP-1c to the Golgi apparatus, where it matures (Yellaturu *et al.*, 2009; Sato, 2010).

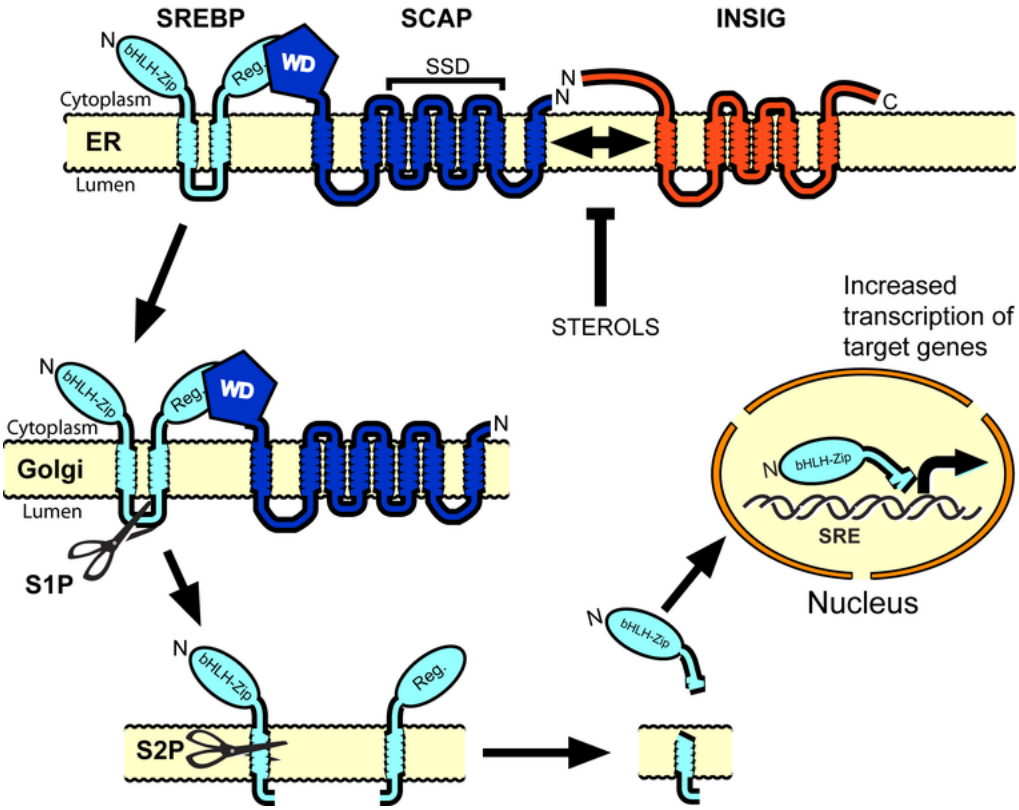


Figure 1.3.1 SREBP activation pathway. SREBP associates with SCAP via the C-termini, and SREBP:SCAP then binds to INSIG, which is retained in the endoplasmic reticulum (ER). When sterol levels decrease, or insulin signalling increases, INSIG and SCAP dissociate and SREBP:SCAP translocates to the Golgi apparatus, where proteolytic cleavage by S1P and S2P releases the mature SREBP protein, which is then able to bind to SREs in the nucleus (Rawson, 2009).

Each of the SREBPs can activate all of their target genes, but with varying affinities and differing effects on transcription. SREBP1 is involved in the lipogenic program, whereas SREBP2 targets genes involved in cholesterol biosynthetic pathways.

Unlike SREBP-2 and 1-a, the expression of SREBP-1c is not regulated by cholesterol (Sheng *et al.*, 1995). SREBP-1c is expression is induced by insulin (Shimomura *et al.*, 1999). This effect on SREBP-1c expression appears to be mediated via the PI3K-signalling pathway. Akt and PKC have both been shown to play a role in this action (Fleischmann & Iynedjian, 2000; Matsumoto *et al.*, 2003). SREBP-1c expression is also induced by Liver X Receptor-α (LXRα) (Repa *et al.*, 2000; Yoshikawa *et al.*, 2001b). LXR is activated by cholesterol derivatives, and the function of its binding with SREBP-1c may be to induce lipogenesis to create cholesterol esters, which would be capable of buffering high cholesterol concentrations (Tontonoz & Mangelsdorf, 2003).

PUFAs can inhibit transcription of SREBP-1c by antagonising ligand dependent activation of LXR (Ou *et al.*, 2001; Yoshikawa *et al.*, 2001b). Interestingly dietary saturated fatty acids appear to have the opposite effect, whereby short-term high fat feeding in mice, induces hepatic SREBP-1c expression along with genes involved in the lipogenic programme, which Lin *et al* believe is effected through the coactivational properties of PGC-1β (SREBP binds with PGC-1β at a domain not present in PGC-α) (Lin *et al.*, 2005). This may be one mechanism by which high-fat feeding could stimulate hyperlipidemia. Inhibition of RIP by PUFAs is a further factor in reducing the abundance of SREBP1c in the nucleus, possibly accounting for the decrease in acetyl-CoA carboxylase (ACC), fatty-acid synthase (FAS), pyruvate kinase (PK) and glucokinase (GK), in hepatocytes (Nakamura *et al.*, 2004; Jump *et al.*, 2005b).

As stated earlier, SREBP-1c is principally involved in regulating the lipogenic program in cells. It does this in concert with various coactivators and with different responses depending on the tissue type and nutritional status.

SREBP-1c induces expression of fatty-acid synthase (FASN); an enzyme responsible for the production of lipids (*de novo* lipogenesis) by conversion of acetyl-CoA and malonyl-CoA into palmitate in adipose tissue (Kim *et al.*, 1998), liver (Foretz *et al.*, 1999) and skeletal muscle (Ducluzeau *et al.*, 2001). SREBP-1c targets hexokinase II (HKII) in adipose tissue and skeletal muscle, where its pattern of expression during fasting and refeeding is similar to that of FASN (Gosmain *et al.*, 2005). HKII catalyzes the phosphorylation of glucose into glucose-6-phosphate (G6P). Deletion of the SRE

site in the HKII promoter, abolishes the inducing effect of insulin on HKII expression in cultured human myotubes (Gosmain *et al.*, 2004). SREBP-1c has been found to increases the flux of the pentose phosphate pathway, through induction of glucose-6-phosphate dehydrogenase (G6PDH) in mouse liver (Shimomura *et al.*, 1998). G6PDH converts glucose-6-phosphate into 6-phosphogluconic acid lactone. This step may have consequences for the activation of carbohydrate response element binding protein (ChREBP), since 6-phosphogluconic acid lactone is later converted into xylulose 5-phosphate (Xu 5-P). Xu 5-P is a substrate for the production of NADPH, and also activates protein phosphatise (PP2A), allowing ChREBP to enter the nucleus. In hepatocytes, SREBP-1c mediates the pro-transcriptional effects of insulin on glucokinase (GK) expression (Bécard *et al.*, 2001; Ferre *et al.*, 2001). SREBP-1c has also been shown to regulate the expression of ACC, and steroyl-CoA desaturase 1 (SCD). Adipose tissue SREBP-1c expression is lower in the obese state, compared with lean controls, but increases with weight loss, possibly due to improved insulin sensitivity. (Kolehmainen *et al.*, 2001; Sewter *et al.*, 2002) Similar decreases in SREBP-1c expression have been observed in the skeletal muscle of diabetic patients, but the picture is more mixed regarding obesity (Sewter *et al.*, 2002) Fasting decreases the abundance of SREBP-1c in mRNA in rat skeletal muscle, and refeeding restores it but this is not linked to the action of insulin. It's targets, HKII and FAS were also correlated with changes in SREBP-1c expression in this study (Commerford *et al.*, 2004).

1.3.2 Carbohydrate Response Element Binding Protein (ChREBP)

ChREBP is another transcription factor belonging to the basic-helix-loop-helix-leucine zipper (bHLH/LZ) family. It binds to carbohydrate response element (ChRE) sequences, which are comprised of two, 5′-CACGTG type E-box motifs separated by 5 base pairs (Yamashita *et al.*, 2001). As its name indicates; ChREBP is responsible for affecting glucose induced changes in gene expression. ChREBP itself is expressed in many tissues, but most highly in metabolically active tissues such as; liver, white and brown adipose and skeletal muscle (Yamashita *et al.*, 2001; Iizuka *et al.*, 2004).

ChREBP is positively regulated by glucose, independently of insulin, in a process whereby, increased glucose is metabolised via the pentose phosphate pathway (PPP). One product of the PPP is xylulose 5-phosphate (Xu 5-P), a metabolite which increases the activity of a protein phosphatase, (PP2A) (Doiron *et al.*, 1996). PP2A, activation allows ChREBP to translocate to the nucleus where it can induce lipogenic gene expression (Kabashima *et al.*, 2003). When blood glucose concentrations are low, ChREBP is localised in the cytosol, but it rapidly translocates to the nucleus when blood glucose concentrations rise. This is the result of dephosphorylation of serine and threonine residues. Ser196 - the target for PKA is dephosphorylated, which permits entry of ChREBP into the nucleus. Ser565 and Thr666 are then dephosphorylated by PP2A, which allows it to bind with DNA and initiate transcription of target genes. ChREBP is negatively regulated by glucagon via cAMP and PKA (Burke *et al.*, 2009), which through phosphorylation prevent DNA binding. PUFAs have an inhibitory effect on ChREBP especially during the consumption of a high fat diet (Uyeda *et al.*, 2002). ChREBP works in partnership with SREBP to regulate lipogenesis; with some targets having binding sites for both TFs (Koo *et al.*, 2001; Rufo *et al.*, 2001). This relationship allows a lipid storage program to be initiated only when both insulin and glucose signalling is coordinated, providing an integrated response (Towle, 2001). Studies using metformin support the idea of synergy, showing that AMPK inhibits the activity of both SREBP and ChREBP (Zhou *et al.*, 2001). ChREBP does not work alone, instead it forms a heterodimer with Max-like protein X (Mlx) to mediate glucose induced gene expression in liver (Ma *et al.*, 2006) Mlx acts to distinguish between E-boxes which are glucose responsive and those which are not (Stoeckman *et al.*, 2004; Dentin *et al.*, 2005b). L-type pyruvate kinase (L-PK) and lipogenic genes such as, ACC and FASN are targeted by ChREBP, to promote fat storage through *de novo* lipogenesis and glycolysis (Dentin *et al.*, 2005b). High fat diets are also capable of suppressing the expression of L-PK and FAS, by expediting the degradation of ChREBP, possibly by the action of PUFAs (Dentin *et al.*, 2005a).

1.3.3 The Peroxisome-Proliferator-Activated-Receptors (PPARs)

The Peroxisome-Proliferator-Activated-Receptors are members of the nuclear receptor superfamily. They have a wide range of targets involved in metabolic regulation. At present three PPAR isotypes (α,γ,δ) (Table 1.3.1) have been discovered, and in common with all ligand activated receptors, they have a ligand binding domain (LBD), activating-function domains (AF) and a DNA binding domain (DBD). After ligand binding occurs, the PPARs form obligate heterodimers with RXR, without which they are unable to bind to DNA. Co-repressor proteins such as SMRT and N-CoR are then released (Yu *et al.*, 2005), and potential co-activators like PGC1α or CREB are recruited. The endogenous ligands for the PPARs are fatty acids and their derivatives; eicosanoids and prostaglandins (Forman *et al.*, 1997; Kliewer *et al.*, 1997). Elevated levels of FFAs activate PPARs and depending on the isoform, the response is different. In PPARs, the DBD binds to a PPAR-response-element (PPRE) in the promoter of the target gene, and transcriptional activation is initiated. The activity of PPARs can also be increased through insulin and AMPK induced phosphorylation (Diradourian *et al.*, 2005).

PPARα is expressed in liver, heart, kidney, intestine, pancreas, skeletal muscle and brown adipose tissue where it induces the expression of genes involved in the transport and β-oxidation of fatty acids and glucose oxidation. It is a molecular sensor of FA's and their derivatives - its natural ligands include polyunsaturated fatty acids. Its action is achieved through its multiple target genes, with it being strongly correlated with increasing the expression of CPT-1a, the rate limiting enzyme in β-oxidation. It is also a regulator of CD36, Lipoprotein lipase (LPL), UCP-3 and potentially PDK4 (Zhang *et al.*, 2004). Human PPARα may target TRB3 expression (in liver) which forms an important part of the insulin signalling chain, TRB3 is an inhibitor of Akt, which positively regulates the cell response to insulin (Koo *et al.*, 2004). Fibrates are a class of drugs which target the PPARα receptor to reduce elevated levels of TAGs and cholesterol, this occurs due to increased β-oxidation and LPL. It has been suggested that since PPARα increases fatty acid oxidation not only in liver

and muscle, but also in the pancreas where it may prevent lipotoxicity, and therefore the progression from insulin resistance to type 2 diabetes (Ravnskjaer *et al.*, 2005).

PPARδ is expressed ubiquitously. Much like PPARα it's activated by PUFAs and eicosanoids and also induces the expression of genes which regulate fatty acid β-oxidation, mitochondrial respiration and oxidative metabolism. It also plays a role in the regulation of HDL concentrations. Research suggests that it is also capable of filling the role of PPARα in PPARα-/- mice. Similarly to PPARα, it is upregulated in response to fasting and exercise. PPARδ is present at higher levels in glycolytic myofibers. Post-translational modifications resulting from signalling proteins such as protein kinase A and MAPK, may phosphorylate PPARδ to increase its activity (Barish *et al.*, 2006).

PPARγ has three main splice variants – PPAR-γ1^{b} expressed ubiquitously at low levels, PPAR-γ2, which is expressed uniquely in adipose tissue and PPAR-γ3 found in macrophages, the small intestine and adipose tissue. PPARγ is most highly expressed as the PPARγ2 isoform in white adipose tissue, where it exerts greatest influence. Its main roles are in adipocyte differentiation, fatty acid transport and storage, and glucose metabolism. Its natural ligands are thought to be long-chain PUFAs, prostaglandins and possibly some phospholipids, and it induces the expression of genes including UCP2 & -3, LPL, FATP, FABP, ACS, GK and GLUT4. Variants in the sequence of the PPARγ gene are not common, but in a few studies SNPs have been linked to obesity, diabetes and hypertension (Meirhaeghe & Amouyel, 2004). PPARγ expression is decreased during starvation in rodents, but increases in response to refeeding. It also appears to play a significant role in the development of diabetes, as discussed earlier in this chapter. PPARγ-/- knockouts are usually lethal, however, heterozygous PPARγ deficient mice, are protected from high-fat diet induced obesity, and insulin resistance (Kubota *et al.*, 1999), through decreases in hepatic an skeletal muscle TG content as a result of increased leptin expression, increased fatty-acid oxidation and decreased lipogenesis. Conversely, agonists of PPARγ such as TZDs have successfully treated hyperlipidaemia and hyperglycaemia, by improving lipid storage in white adipose tissue (and reducing

storage in liver and skeletal muscle) – with the side effect of weight gain (Yki-Jarvinen, 2004). A recent study has shown that obesity induced by high fat feeding in mice, activates cyclin dependent kinase 5 (CDK5), which phosphorylates PPARγ, and dysregulates the expression of target genes – including the insulin sensitising adipokine, adiponectin (Choi *et al.*, 2010).

Table 1.3.1 PPAR isotypes. PPAR isotypes, with their corresponding tissue distribution, ligands metabolic function, target genes and potential disease interactions.

Peroxisome-Proliferator-Activated Receptors (PPARS)						
Isotype	**Active state**	**Primary Tissues**	**Ligands**	**Function**	**Targets**	**Related disease process**
PPARα	Post absorptive	Liver, muscle, heart	FFAs. Fibrates	Fat oxidation	FATP, acyl-CoA oxidase, cytochrome P450, CPT1	Dyslipidemia, Diabetic cardiomyopathy
PPAR γ	Postprandial	Adipose, muscle, heart, macrophages	FFAs. TZDs	Fat storage	CD36, LPL,GLUT4, PEPCK, acyl-CoA synthase	Insulin resistance, obesity, NAFLD, LVH
PPARδ	Movement	(Ubiquitous), muscle, adipose	FFAs, prostaglandins	Energy balance	MCAD, CPT1,UCP3	Dyslipidemia, obesity

1.3.4 Peroxisome-proliferator-activated-receptor-γ Co-activator-1α (PGC1α)

Peroxisome-proliferator-activated-receptor-γ Co-activator-1α, is a transcriptional coactivator which, despite its name, is a coactivator of all three PPARs. Its main function is to regulate mitochondrial biogenesis, and hence it is more abundant in the metabolically active tissues, such as skeletal and cardiac muscle and brown adipose tissue, with lower levels found in liver and white adipose tissue. It is induced by cold exposure via the β-adrenergic/cAMP pathway. PPAR Gamma co-activator 1-α (PGC-1α), is part of a family of transcriptional co-activators which induce the expression of genes involved in a number of metabolic processes. These include; adaptive thermogenesis, carbohydrate and fatty acid oxidation and mitochondrial biogenesis. Roles have also been suggested for PGC-1α involvement in hepatic gluconeogenesis and glucose uptake in skeletal muscle through its interaction with FOXO1 (Oberkofler *et al.*, 2003; Puigserver *et al.*, 2003). Coactivators such as PGC-1α do not bind to DNA but affect their targets via interactions with nuclear receptors

such as PPARS α,δ and γ, by increasing their ability to initiate transcription. PGCs can act by docking to specific receptors alone, but can also recruit more co-activators such as CBP/p300 and p160/SRC-1 (Puigserver & Spiegelman, 2003). PGC-1α has been demonstrated to upregulate PDK4 expression in C2C12 myotubes. Gel shift assays revealed that it was bound to the estrogen-related receptor α (ERRα) (Wende *et al.*, 2005). PGC1α induces PDK4 and PDK2 in rat hepatocytes, potentially decreasing PDC flux, reducing the conversion of pyruvate into acetyl-CoA and into oxaloacetate and gluconeogenesis instead (Ma *et al.*, 2005).

1.3.5 The Retinoid X Receptors (RXR)

The Retinoid X Receptor (RXR) also forms part of the nuclear receptor superfamily of transcription-factors which are ligand-activated. The three receptor subtypes are known as, α, β, & γ. The isotypes are encoded by different genes, which through alternative splicing produce a two isoforms each (α1,α2 etc). As the names suggests, of 9-*cis* retinoic acid (RA) coactivates RXR. They were first discovered to bind heterodimerically with the thyroid receptor (TR) and RARs, and later discovered to dimerize with pregnane X receptor, farnesoid X receptor, liver X receptor, constitutively activated receptors (CAR) and PPARs (Szanto *et al.*, 2004). Evidence exists that shows RXR to be capable of activating PPAR targets as a homodimer binding to PPRE sites in gene promoters (IJpenberg *et al.*, 2004).. RXRα is found in liver, kidney, epidermis and intestine, RXRβ is found ubiquitously, but RXRγ is restricted to skeletal muscle, and the pituitary gland (Mangelsdorf *et al.*, 1992). As Germain *et al* have suggested, the fact that RXR is an obligate heterodimer for many nuclear receptors, the number of targets which they regulate at the transcriptional level is vast (Germain *et al.*, 2006). As a result of the myriad binding targets, most research has focussed on the binding partners of RXR.

1.3.6 Liver X Receptors (LXR)

LXRs are another class of nuclear receptors related to RXRs and PPARs. There are two LXR genes, α and β, transcribed from different loci. LXRα is found in liver, adipose tissue, macrophages, bone, intestine and spleen, whilst LXRβ is expressed ubiquitously. LXRs bind to the LXR response element (LXRE) which has the consensus sequence TGGTCACTCAAGTTCA. LXRs are activated by oxysterols, and are involved in cholesterol homeostasis, in conjunction with SREBP1. It regulates a range of metabolic genes, due in part to its promiscuous nature and ability to dimerize with other receptors including RXR. These targets include FAS, LPL, CYP7A1, APOE, ABCs, SREBP-1c and LXR (Repa *et al.*, 2000; Chiang *et al.*, 2001; Laffitte *et al.*, 2001; Yoshikawa *et al.*, 2001a; Zhang *et al.*, 2001; Joseph *et al.*, 2002a). Activation of LXR with the synthetic ligand GW3965 improves glucose tolerance in murine models of obesity and insulin resistance, with reduced gluconeogenesis, and improved glucose uptake – principally by the suppression of hepatic PGC1α, glucose-6-phosphatase and PEPCK. In adipose tissue it increases the expression of GLUT4 (Laffitte *et al.*, 2003).

Mitro *et al* reported that D-glucose and D-G6P were direct agonists of LXR in the liver, which activated a programmer of hepatic lipogenesis and reduced gluconeogenesis (Mitro et al., 2007). This has been challenged more recently, by experiments in mice showing that ChREBP but not LXR are required for the induction of glucose regulated genes in mouse liver (Denechaud *et al.*, 2008). PPARs suppress SREBP-1c expression by inhibiting the binding of RXR:LXR to the SREBP-1c promoter (Yoshikawa *et al.*, 2003). It may be that the ratio of available RXR binding with either PPARs or LXR determines the program of lipogenesis or β-oxidation, however these findings are yet to be replicated *in vivo*.

1.4 Aim of thesis

The field of nutrigenomics has developed to investigate the questions surrounding how metabolic gene expression adapts to the changing environment, and how its dysregulation may contribute to disease states. As this introduction has shown, the diverse regulatory mechanisms compliment and compete with one another to achieve energy balance. Transcription is a major regulator of energy balance and peripheral substrate flux. Experimental observation of metabolic gene expression coupled with pathway analysis offers a powerful way of understanding how these systems operate.

This aim of this thesis is to investigate how fuel substrates interact with gene expression to generate a particular phenotype and how gene expression adapts to alterations in substrate availability. The major themes of this thesis are;

1. Changes in metabolic gene expression during a 10-week low-energy diet of varying fat content in obese subjects with high or low postprandial fat oxidising capacity.
2. Differences in the global gene expression profiles of obese subjects with high- or low-postprandial fat oxidation rates.
3. Acute hyperglycaemia induces the expression of lipogenic genes in human primary myotubes.
4. Increasing PDC flux, independently of insulin signalling does not affect gene expression in human skeletal muscle.

Chapter 2

Molecular Biology: Materials & Methods

2.0 Molecular Biology Materials and Methods†

2.1 Human study design and protocol

The human studies described in this thesis (section 2.1.1 – 2.1.4) the author was provided with total RNA for subsequent quantification by RT-QPCR and total protein extracts for quantification of protein expression by Western blotting, and therefore acknowledges the assistance of the following in performing the human studies.

2.1.1 Fat oxidising capacity influences gene expression changes during 10-week low-fat or high-fat low energy diets in obese subjects (Tantip Boonsong, Professor Ian Macdonald, Dr Andrew Bennett, NUGENOB) (See Chapter 3).

2.1.2 Transcriptional Profiling of Adipose Tissue in Obese Subjects with High- or Low-Postprandial Fat Oxidising Capacity (Hui Shi, Christopher Medway, Kevin Morgan, Professor Ian Macdonald, Dr Andrew Bennett, NUGENOB) (See Chapter 4).

2.1.3 Effects of high-glucose treatment on metabolic gene expression in Human primary myotubes. (Roya Jadidi-Babaei, Dr Kostas Tsintzas, Professor Ian Macdonald, Dr Andrew Bennett) (See Chapter 5).

2.1.4 Skeletal muscle metabolic gene expression is not affected by dichloroacetate mediated modulation of substrate utilisation (Dr Andrew Bennett, Dr Kostas Tsintzas, Professor Ian Macdonald, Dr Nandini Seevaratnam) (See Chapter 6).

† Detailed study protocols and methods are included in individual chapters

2.2 Molecular Biology

2.2.1 Isolation of RNA

Total RNA was extracted from human muscle tissue (stored under liquid nitrogen) by placing ~50 mg into cryovial tubes, to which 800 µL TRIzol® (Invitrogen) and 20 µL glycogen (10µg/µL) were added. This was homogenised (Polytron, USA) at speed 2.5 for 30 seconds. After incubation at room temperature for 5 minutes the solution was transferred to a 1.5 ml RNase-free Eppendorf tube. Following this 156.8 µL of Chloroform and 3.2 µL of Isoamyl alcohol were added and the tube was briefly vortexed and incubated at room temperature for 2 minutes. The sample was centrifuged at 12, 000g at 4°C for 15 minutes and the aqueous phase (approx 500 µL) transferred to a clean 1.5 ml Eppendorf tube. 400 µL isopropanol was added and the sample placed at -20°C overnight or for a minimum of 4 hours. Subsequently the samples were centrifuged at 12,000g at 4°C for 15 minutes. The supernatant was then removed and the RNA pellet air dried for 5 minutes. The pellet was washed with 800 µL 75% EtOH by vortexing, and then centrifuged at 10,000g for 10 minutes. The supernatant was then removed, and the pellet air dried for 5 minutes. The RNA pellet was finally re-dissolved in 30 µL of RNase-free water and stored at -80°C.

2.2.2 Reagents

Glyoxal reaction mixture

1x BPTE buffer

1M deionised glyoxal

4.8% (v/v) glycerol

60% (v/v) DMSO

20% (w/v) ethidium bromide

BPTE electrophoresis buffer

1mM EDTA, pH 8.0

10mM PIPES (piperazine-1,4-bis[2-ethanesulphonic acid], pH 6.5

30mM Bis-Tris (bis [2-hydroxyethyl] iminotris [hydroxymethyl] methane)

TBE (Tris Borate EDTA) buffer

0.09M Tris-HCl, pH 7.5

0.09M Boric acid

0.01mM EDTA, pH 7.5

2.2.3 Agarose gel electrophoresis of total RNA (Glyoxal/DMSO method)

To assess RNA integrity, RNA samples (1-2 µg) were mixed with 10 µL of glyoxal reaction mixture, and then incubated at 55°C for 1 hour and placed on ice for 10 minutes. 2µL of loading dye was added to the sample, which was then loaded into the wells of a 1.5% agarose gel (w/v) made up in BPTE buffer. The gel was run at 90 V (5 V/cm). The RNA 18S and 28S bands were visualised with a UV transilluminator, and the photograph recorded using Genesnap software.

2.2.4 Agarose gel electrophoresis of cDNA (Ethidium Bromide method)

A 1% (w/v) agarose gel was produced using 0.5g of agarose in 50 ml of TBE (heated in microwave until dissolved) after allowing it to cool to 60°C, 1 µL of ethidium bromide (10mg/mL) was added to the solution and mixed by gently swirling the flask. The combs were inserted into the tank and the gel was poured slowly, following which the gel was left at room temperature for 40 minutes. Once set TBE buffer was poured into the tank until the gel was submerged by 4mm depth. 10 µL of the PCR product was mixed with 2 µL 6x Loading dye prior to being pipetted into the wells. The gel was run at 100V (5V/cm). Once the gel had finished running it was placed under UV light and photographed using GeneSnap software.

2.2.5 Reverse Transcription (RT) 1.

cDNA was produced from human tissues by the reverse transcription of total RNA in (section 2.1.4). RNA samples were firstly quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific, Delaware, USA), and then diluted to a concentration of 50 ng/µL in RNase-free water. 10 µL of RNA and random primers (Invitrogen) at 0.5µg/µL concentration were added to 0.5 ml PCR tubes along with 1 µL of dNTP's (Invitrogen) at a concentration of 10 mM. 1 µL of RNase-free water was added to give a total volume of 13 µL. The tubes were briefly vortexed and centrifuged before being placed into a thermocycler with a heated lid at 65°C for 5 minutes then placed on ice for at least 2 minutes. Whilst on ice 4 µL of 5 X First-strand buffer (Invitrogen), 1 µL of RNase-inhibitor (40u/µL), 1 µL of Superscript™ III RT (200u/µL) (Invitrogen) and 1 µL of 0.1M DTT were added to the primer:RNA mix. The tubes were then incubated at 25°C for 5 minutes, 50°C for 1 hour, 70°C for 15 minutes and then cooled to 4°C. cDNA was stored at -20°C until RT-QPCR analysis.

2.2.6 Reverse Transcription (RT) 2.

The Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) kit was used to produce cDNA in sections 2.1.1-2.1.3. 1 µg of RNA was added to a thin-walled 0.2 ml PCR tube along with 10 µL 2X RT Reaction Mix, 2 µL RT Enzyme Mix and RNase-free water to 20 µL. The tube was gently mixed and then incubated at 25°C for 10 minutes, 50°C for 30 minutes and 85°C for 5 minutes before cooling to 4°C and storing at -20°C. Thermocycling was carried out on a Geneamp PCR System 9700 with a heated lid.

2.2.7 Polymerase Chain Reaction (PCR)

To give a total volume in of 25 µL in a single PCR reaction;

Reagents	Volume
cDNA	5 µL
5 x PCR Buffer (Promega)	2.5 µL
Forward Primer (10 µM)	0.75 µL
Reverse Primer (10µM)	0.75 µL
dNTPs (10mmol)	0.5 µL
Taq Polymerase (Promega)	0.25 µL
H_2O	15.25 µL

The tubes were flick mixed and briefly centrifuged. They were then placed on a thermocycler (with heated lid) at 95°C for 50 minutes, 40 X (95°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes) and finally 72°C for 5 minutes. The product size and specificity were assessed by using agarose gel electrophoresis and ethidium bromide staining.

2.2.8 Analysis of mRNA expression by TaqMan® (Applied Biosystems, Foster City, CA USA) real-time quantitative PCR

Reaction set up

Each reaction was in a final volume of 25uL (see below) but a series of master mixes were made up to ensure reproducibility.

Reagents	Volume per reaction	Final concentration
TaqMan® Universal PCR Mastermix	12.5uL	-
Forward primer (10 µM)	0.75uL	0.3uM
Reverse primer (10 µM)	0.75uL	0.3uM
Probe (dual labelled) (10 µM)	0.5uL	0.2uM
cDNA	5uL	-
Water (molecular grade)	5.5uL	-
Total volume	**25 uL**	

The reactions were carried out using; ABI PRISM 7000 sequence detection system, ABI PRISM 7700 sequence detection system and an ABI StepOne Plus (Applied Biosystems, UK). 5µL of RNase-free water was used as a negative/No Template Control (NTC). Thermo-cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 x (95°C for 15 seconds, 60°C for 1 minute). Once the reaction had been completed, the mRNA was quantified using the standard curve method. The standard curve consisted of two-fold serial dilutions of cDNA reverse transcribed from a known quantity of RNA. All standards, samples and negative controls were assayed in triplicate to ensure accurate results. The threshold cycle (Ct Value) is the point at which fluorescence rises to a point considered statistically significant above the baseline values. A standard curve was then constructed by plotting Ct values versus the logarithm of the serial cDNA dilutions. The efficiency (E) of the reaction was calculated with the equation.

$$E = 10^{(-1/slope)} - 1$$

Any assay which did not have a standard curve with a slope between -3.0 and -3.6 (90-100% efficient) was rejected as were any results where the values for the triplicates had a range greater than 0.5 Ct. The control genes used during experiments were Hydroxymethylbilane Synthase (HMBS), and 18S. All values were normalised by dividing through these genes.

Table 2.2.1 Oligonucleotide sequences for probes and primers

Gene	Sequences (5' -→ 3')	Amplicon size (bp)	Gen Bank Accession No.	Origin
18S	FWD CGGCTACCACATCCAAGGAA PROBE TGCTGGCACCAGACTTGCCCTC REV GCTGGAATTACCGCGGCT	188	M10098	Dr A Bennett
α-ACTIN	FWD GAGCGTGGCTACTCCTTCGT PROBEACCACAGCTGAGCGCGAGATCGT REVGTAGCACAGCTTCTCCTTGATGTC	72	NM_001100	Dr K Tsintzas
ACC2	FWD CACACTCTGCCCAAGACACC PROBE CCCCAAAGATGCCGGTCGGC REV TTCTGGTGGGAGGGTGGTAG	101	U89344	Dr T Boonsong
apM1	FWD TGCAGTCTGTGGTTCTGATTCC PROBE GGCTCAGGATGCTGTTGCTGGGA REV TTGAGTCGTGGTTTCCTGGTC	109	NM_004797	Dr T Boonsong
ATGL	FWD CCAACACCAGCATCCAGTTC PROBE CTCTTCCCGCCGGAGCCCCT REV TATCCCTGCTTGCACATCTCTC	103	NT_035113	Dr T Boonsong
CPT1	FWD TTTGGCCCTGTAGCAGATGAT PROBE TCGTGTTCTCGCCTGCAATCATGT REV ACTTGCTGGAGATGTGGAAGAAG	85	Y08683	Dr K Jewell
HKII	FWD AAGTTCTTGTCTCAGATTGAGAGTGACT PROBE CTGCAACACTTAGGGCTTGAGAGCACCTG REV CAGTGCACACCTCCTTAACAATG	121	NM_000189	Dr T Boonsong
HMBS	FWD ---- PROBE ---- REV ----	--	--	Applied Biosystems, UK
HSL	FWD GATGCTTCTATGGCCGCTG PROBE TTCCAGTTCACGCCTGCCATCCG REV GACACCAGCCCAATGGAGA	85	BC070041	Dr T Boonsong
MCAD	FWD GCCGTGACCCGTGTATTATTG PROBE TTCGGGCGATGCTGCAGGGT REV CGATTGGCTTTTGTATGC TGTG	133	MN_000016	T Boonsong
PDK2	FWD CATCATGAAAGAGATCAACCTGCTT PROBE CCGACCGAGTGCTGAGCACACCC REV CAGGAGGCTCTGGACATACCA	109	NM_002611	Dr K Tsintzas
PDK4	FWD CAAGGATGCTCTGTGATCAGTATTATTT PROBE CATCTCCAGAATTAAAGCTTACACAAGTGAATGGA REV TGTGAATTGGTTGGTCTGGAAA	90	NM_002612	Dr K Tsintzas
PGC-1α	FWD GGTGCAGTGACCAATCAGAAATAA PROBE ATCCAATCAGTACAACAATGAGCCTTCAAACATAT REV TTGCCTCATTCTCTTCATCTATCTT	90	NM_013261	Dr K Tsintzas
PPARα	FWD GCTTCCTGCTTCATAGATAAGAGCTT PROBE AGCTCGGCGGCACAACCAGCA REV CACCATCGCGACCAGATG		NM_005036	Dr K Tsintzas
PPARδ	FWD TGCGGCCATCATTCTGTGT PROBE ACCGGCCAGGCCTCATGAACG REV CAGGATGGTGTCCTGGATAGC		NM_006238	Dr K Tsintzas
SREBP-1C	FWD GGAGGGGTAGGGCCAAC PROBE CGCGGAGCCATGGATTGCAC REV GTCAAATAGGCCAGGGAAGTC	150	NM_004176	Dr T Boonsong
UCP2	FWD CGTCTCCCACCCATTTTCTATG PROBE CCAAGGGGATCGGGCCATGA REV GGAATCCGGCTTTGTAAG GTCT	135	BC011737	Dr T Boonsong
UCP3	FWD CTGGACTACCACCTGCTCACT PROBE AGGATCCCAAACGCAAAAAGGAGG REV AGGTTACGAACATCACCACGT		NM_003356	Dr K Tsintzas
SREBP1a	FWD GCGAGCCGTGCGATCT PROBE CGCTGCTGACCGACATCGAAGACA REV CTGTCTTGGTTGTTGATAAGCTGAA		NM_001005291	P Tisdale

2.2.10 Reduction of ribosomal RNA by Invitrogen Ribominus module

RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) Version C

2.2.11 Affymetrix HuEx 1.0 ST Array

GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual Version 4

2.3 Primary cell culture (Human myotubes)

Human muscle biopsy material was washed 3 times in 10ml of PBS, and then processed into a 'tissue mash' using a scalpel (no.22 blade). The sample was then transferred to a universal tube containing a 'flea' and 5ml of 1x trypsin for incubation at 37ºC for 15 minutes. The supernatant was transferred to a universal tube and neutralised using 5ml of media (Ham's F-10, streptomycin, penicillin and foetal bovine serum), then centrifuged at 1700 rpm for 5 minutes. The supernatant was removed and satellite cells were resuspended in 1-2 ml of media, by gentle aspiration with a pasteur pipette. 1ml of satellite cells was pipetted into each gelatinised flask (washed with PBS after 24 hours) and grown at 37ºC and 5% CO2. Media was changed every 2 days for ~5weeks until the cells had differentiated into a dense multinucleated myotube network, at which point they were treated.

2.4 Protein gel electrophoresis and Western blotting

2.4.1 Reagents

10% Ammonium persulfate

0.1g of Ammonium persulfate was dissolved in 1ml of distilled water immediately prior to use.

SDS-PAGE gel solutions (for 2 gels)

10% Separating gel	**(20ml)**
30% Acrylamide/bisacrylamide (Severn Biotech Ltd.)	6.68 ml
2M Tris HCl (pH 8.8)	3.76 ml
10% SDS (w/v)	0.2 ml
TEMED	20 µL
Distilled water	9.18 ml
10% Ammonium Persulfate	160 µL

5 % Stacking gel	**(10ml)**
30% Acrylamide/bisacrylamide (Severn Biotech Ltd.)	1.7 ml
1M Tris HCl (pH 6.8)	1.26 ml
10% SDS (20g/200ml)	100 µL
TEMED	10 µL
Distilled water	6.84 ml
10% Ammonium Persulfate	100 µL

SDS-PAGE running buffer

1.92 M Glycine

0.25 M Tris base

10% (w/v) SDS

Transfer buffer (for western blotting)

1.92 M Glycine

0.25 M Tris

20% (v/v) Methanol

Ponceau Red

0.1% (w/v) Ponceau S (3-hydroxy-4[2-sulpho-4-(4-sulphophenylazo)-phenyl-azo]-2,7-napthalenedisulphoric acid) in 5% (v/v) acetic acid.

TBS

1.2g Tris Base

8.75g NaCl

pH adjusted to 7.5 and made up to 1 L with distilled H_2O

TBS-T

TBS

0.1% (v/v) Tween™20

Blocking buffer

TBS-T

5% (w/v) non-fat dry milk (Marvel)

Stripping buffer

Biorad, UK

Protein extraction from human tissue

Trizol reagent (Invitrogen, UK)

2x Protein sample loading buffer

1 M Tris (pH 6.8)

20% (v/v) Glycerol

4% (w/v) SDS

100 mM DTT

Bromophenolblue (0.01% w/v)

Protein solubilisation buffer

9M Urea

50 mM Tris

4% SDS

Protein quantification

Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

2.4.2 SDS-PAGE

The separating gels were mixed as described in 2.4.1. The separating gel was then cast in a Bio-Rad (Hercules, CA) miniature slab gel apparatus (Mini-Protean) and overlaid with water-saturated butanol and allowed to polymerise fully for 30 minutes. After the gel had set, the butanol was removed and the gel surface rinsed with distilled water. 3M filter paper was used to remove any excess water. The stacking gel was mixed and then poured on-top of the separating gel, after which the comb was inserted between the plates. A further 30 minutes were allowed for polymerisation of the stacking gel. 50 µg of protein was mixed with 2x protein loading buffer to a total volume of 50 µL and then boiled for 4 minutes. The protein samples were then loaded into the wells of the stacking gel, with 10 µL of SeeBlue® Plus2 Pre-stained Standard (Invitrogen) molecular weight markers loaded at either end of the gel. The proteins were separated using the Bio-Rad Mini-Protean tanks filled with SDS-PAGE running buffer. The samples were run at 40mA for 120 minutes.

2.4.3 Electrotransfer

The samples were transferred to Hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare) (pre-soaked in 100% methanol for 15 seconds and then distilled water for 2 minutes). 3M filter paper and sponges were pre-soaked in transfer buffer. Electrotransfer was carried out in the Bio-Rad mini-protean, with ice filled inserts at 200 mA for 120 minutes. Protein bands were visualised using Ponceau S and subsequently destained by rinsing with distilled water. Non-specific protein binding was reduced by incubating the membrane in 5% blocking buffer on a shaker for 1 hr at room temperature. Primary antibodies were diluted in 1% blocking buffer, and then applied to the membrane and incubated overnight at 4°C on a roller. After the incubation was completed, the membrane was washed with 2 x 10 minute washes in TBS-T and 2 X 10 minute washes in 1% blocking buffer. The membrane was then incubated with horseradish peroxidise-conjugated (HRP) secondary antibody diluted at a 1:2000 ratio with 1% blocking buffer for 1 hr at room temperature. The membrane was then washed for 5 x 10 minutes in TBS-T, and rinsed with distilled water. The antigen:antibody complexes were then visualised by soaking the membrane with either ECL™ or ECL™ Plus (GE healthcare), and placing it into a cassette and exposing it to Amersham Hyperfilm ECL (GE Healthcare). Band intensities were quantified by optical densitometry using Bio-rad, Quantity One software (Bio-Rad, Hercules, CA).

Table 2.4.1 List of antibodies

Protein	Primary Antibody	Secondary antibody	Tissue	Amount of protein loaded per well (µg)
αActin	Rabbit anti- αActin antibody. Diluted at 1:2000 with 1% blocking buffer. (Sigma-Aldrich® #A2066)	Swine polyclonal anti-rabbit Ig. Conjugated with HRP. Diluted at 1:2000 with 1% blocking buffer. (Dako #P0217)	Skeletal muscle	50
Akt	Rabbit polyclonal antibody. Anti Akt. Diluted at 1:1000 with 1% blocking buffer. (Cell signalling TECHNOLOGY® #9272)	Swine polyclonal anti-rabbit Ig. Conjugated with HRP. Diluted at 1:2000 with 1% blocking buffer. (Dako #P0217)	Skeletal muscle	50
Phospho-Akt	Mouse monoclonal IgG2b antibody. Anti-phospho(Ser^{473}) Akt. Diluted at 1:1000 with 1% blocking buffer. (Cell signalling TECHNOLOGY® #4051)	Goat polyclonal anti-mouse Ig. Conjugated with HRP. Diluted at 1:2000 with 1% blocking buffer. (Dako #P0447)	Skeletal muscle	50

Chapter 3

Fat oxidising capacity influences gene expression changes during 10-week low-fat or high-fat low energy diets in obese subjects

3.0 Fat oxidising capacity influences gene expression changes during 10-week low-fat or high-fat low energy diets in obese subjects

3.1 Introduction

Metabolic disorders such as obesity, type-2 diabetes and atherosclerosis have become much more prevalent in the global population during recent decades. As a result there has been extensive research into how various diet regimes can influence weight loss in obese individuals (McManus *et al.*, 2001; Astrup *et al.*, 2002; Schoeller & Buchholz, 2005), and yet whilst there is widespread agreement between clinicians and researchers that reduced energy intake is beneficial in reducing body-mass index (BMI) (Labib, 2003) there remains considerable debate as to what constitutes the most effective and safe dietary composition, including among other aspects the ratio of carbohydrate to fat (Acheson, 2010). Both genes and the environment influence metabolic disorders, such as obesity. Differences between individuals at the transcriptomic level, most likely as a result of interaction between the genes and the environmental influences are increasingly being recognised as key elements in maintaining energy balance. Therefore further investigation, into the transcripts where these effects are produced, is warranted.

Hypo-energetic diets are known to influence the transcription of metabolic genes. Previous studies using subjects from the NUGENOB trial (3) have examined how different diets affected changes in gene expression (Petersen *et al.*, 2005; Viguerie *et al.*, 2005; Jocken *et al.*, 2007; Capel *et al.*, 2008; Corpeleijn *et al.*, 2009), but none looked at this within the context of fat oxidising capacity. The differences in fat oxidation rates between individuals may be an important factor in determining weight-loss during energy-restricted diets of varying carbohydrate and fat content. Examining how fat oxidising capacity is related to variations in adipose tissue gene expression, and whether this might be altered by dietary composition would provide a more comprehensive understanding of nutrient-gene interactions, which could in turn relate to differences in weight loss and phenotypic characteristics.

Here we performed a study on obese subjects with different baseline postprandial fat-oxidising capacities, and different dietary composition during weight loss. A candidate gene approach was utilised, with transcripts chosen on a functional basis (some of which had been assessed in previous NUGENOB studies (Sørensen *et al.*, 2006; Jocken *et al.*, 2007)). The genes studied represented lipolysis; adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), lipogenesis; sterol-regulatory-element-binding protein-1c (SREBP-1c), fatty acid oxidation; medium-chain acyl-coenzymeA dehydrogenase (MCAD), energy expenditure; uncoupling protein-2 (UCP2) and glucose regulation/fatty acid catabolism; adiponectin (apM1). Our analysis utilised Real-time quantitative PCR (RT-qPCR), combined with a general linear model univariate analysis to see how differences in baseline fat-oxidising capacity between obese individuals might reflect disparities in adipose tissue gene expression, weight loss and other metabolic parameters when consuming low energy diets of varying fat content.

3.2 Materials and methods

Study protocol and selection of individuals

771 obese Caucasian Europeans (579 women) (age 20-50 yr, BMI ≥ 26 kg/m^2) participated in the NUGENOB study, funded by the European Union, which is described by Petersen et al. (Petersen *et al.*, 2005). Subjects in this study were randomly assigned to either a high-fat, low carbohydrate (HF) or low-fat, high carbohydrate (LF), 10-week hypoenergetic diet (600 kcal less than their estimated daily requirement). The diet contained 40–45% or 20–25% fat, and 40–45% or 60–65% carbohydrate in the HF and LF diets respectively, with the remainder being derived from protein.

For the purposes of this study, 200 individuals were drawn from an original group of 648 individuals who completed the diet. They were chosen on the basis of their respiratory quotient (RQ) after a high-fat test meal during the pre-weight loss clinical evaluation. The postprandial fat oxidation data (iAUC) was used to rank them into two distinct and non-overlapping groups, with the top 100 referred to as the high

(HX) and the bottom 100 the low (LX) –fat oxidising groups, respectively. The subjects were then divided into two further groups; those on HF diet and those on LF diet. This gave four groups (n=50 in each); high postprandial fat oxidisers on a high-fat diet (HXHF), high postprandial fat oxidisers on a low-fat diet (HXLF), low postprandial fat oxidisers on a high-fat diet (LXHF), and low postprandial fat oxidisers on a low-fat diet (LXLF). Half of the subjects in each fat ox/diet group were then randomly assigned to either a preliminary study (see below), or to the main/present experiment.

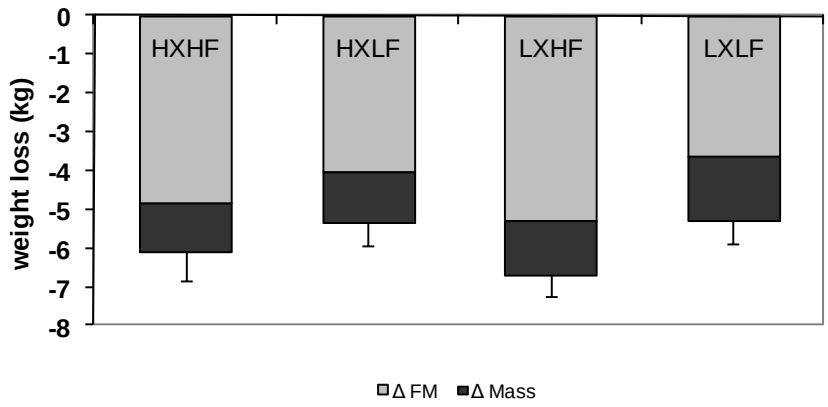


Figure 3.2.1 Weight loss during the 10-week diets. Mean values are shown in each histogram, displaying Δmass and Δfat mass (FM). Error bars are S.E. HX, high fat oxidising; HF, high-fat diet; LX, low fat oxidising; LF, low-fat diet.

Preliminary analysis

A preliminary (unpublished) study with 100 subjects (25 in each of the 4 groups), also drawn from the NUGENOB cohort, using identical criteria to the present investigation, had revealed that SREBP-1c was significantly down regulated post-diet in HXLF, but there were no other changes in gene expression *per se* during weight loss. However, statistical analysis had shown associations between the expression of SREBP-1c and both FFA and HOMA-IR, and between HSL and energy intake, while ATGL expression was affected by changes in HOMA-IR.

The present study describes the use of the second set of individuals (without re-use of the first set) to examine the potential relationships between phenotypic

(metabolic) characteristics and adipose tissue gene expression changes during weight loss in which we refined our experimental techniques by using improved reagents, adjusted dilutions, triplicate RT reactions and a more sensitive RT-qPCR machine (as described in the methods below), to investigate these relationships in the remaining subjects.

Anthropometry and metabolic rate

Body mass was measured using calibrated scales. Waist circumference was measured with the participant wearing only non-restrictive underwear. Body height was measured using a calibrated stadiometer. Fat mass and fat-free mass were assessed by multifrequency bioimpedance (Bodystat®; QuadScan 4000, Isle of Man, British Isles). Resting metabolic rate and substrate oxidation were measured via a ventilated hood system (Petersen *et al.*, 2005; Blaak *et al.*, 2006).

Biochemical analyses

Venous blood samples were drawn after an overnight fast of 12 h, following a 3-day period when subjects had been instructed to avoid excessive physical activity or alcohol consumption. Subjects rested in the supine position for 15 min prior to the procedure. Blood metabolites were assayed using techniques previously described (Petersen *et al.*, 2005; Viguerie *et al.*, 2005).

RNA isolation

After an overnight fast adipose tissue biopsies were taken from subcutaneous abdominal fat (~1 g), both before and after the dietary intervention (Petersen *et al.*, 2005). The adipose tissue specimens were stored in RNA Later solution, at -80°C. RNA was extracted using the RNeasy total RNA Mini kit (Qiagen, Courtaboeuf, France) at the INSERM laboratory in Toulouse. The quality of the RNA was assayed using a 2100 Bioanalyser (Agilent Technologies, Massy, France).

mRNA quantification by Real Time – Quantitative PCR (RT-qPCR)

cDNA was synthesized from 1µg of RNA (after DNase treatment), using Superscript III Supermix for qRT-PCR (Invitrogen) in accordance with the protocol; the reaction was carried out on a GeneAmp PCR System 9700. Probes and primers for TaqMan® RT-qPCR were designed using Primer Express V2.0 software (Applied Biosystems, UK) and manufactured by MWG (Germany). The reaction was carried out in a 96-well Micro-amp plate, utilising both an ABI Prism 7700 Sequence Detection System and an ABI StepOne Plus (Applied Biosystems, UK). Probes and primers were used at a final concentration of 10µM in a reaction volume of 25µL, containing 5µL of cDNA. Assays were performed in triplicate with the average threshold cycle (Ct) values normalised using 18S. The Ct values for 18S did not change significantly between time-points, nor was there any bias between machines. The slope of the standard curve was maintained between -3.1 and -3.6.

Statistical Analysis

Statistical analyses were performed using SPSS V. 15 (SPSS, Chicago, IL). Due to missing samples only 89 subjects were assessed. Changes in phenotypic parameters and mRNA expression levels were compared using a paired Student's *t*-test and ANOVA. General linear model univariate analysis was used to study the effect of changes in phenotypic parameters on gene expression, for each of the dietary/fat-oxidising groups. Values given are means ± S.D.

3.3 Results

Phenotypic measurements

All groups showed a significant reduction in body weight; HXHF 6.08 ± 3.33 kg (n=19), HXLF 5.32 ± 2.96 kg (n=23), LXHF 6.69 ± 3.03 kg (n=27) and LXLF 5.29 ± 2.75 kg (n=20), with no significant difference between groups (Figure 3.2.1). This mass was mainly lost from body fat (HXHF 4.83 ± 2.42 kg, HXLF 4.03 ± 2.51 kg, LXHF 5.29 ± 2.39 kg and LXLF 3.65 ± 2.17 kg). Significant reductions for all groups were seen in fat-free mass, waist circumference, energy intake and leptin concentrations. Serum insulin concentration fell over the dietary intervention period by 2.24 ± 4.91 µU ml^{-1} in HXLF and 2.84 ± 3.69 µU ml^{-1} in LXLF, but was unchanged with the high-fat diets. Cortisol increased significantly in LX, and showed a trend for increasing in HXHF. IGF fell in HXHF ($P<0.05$) but was unchanged in other groups. A significantly lower value for HOMA-IR was observed after the dietary intervention in LXLF, indicating improved insulin sensitivity, but the smaller decreases in the other groups were not significant - although there was a trend in HFLX. FFA showed a trend for decreasing in HXLF p=0.054. (Table 3.3.1)

Table 3.3.1 Metabolic and phenotypic measurements for high- and low-postprandial fat oxidising groups, assigned to high or low fat diets for 10 weeks. Values are mean ± S.D. FFM, fat-free mass; FM, fat-mass; FFA, free fatty-acids; IGF, insulin-like growth factor; HOMA-IR, homeostasis model assessment -insulin resistance.

	High postprandial fat oxidisers -1						**Low postprandial fat oxidisers - 2**					
	High fat diet (n=19)			*Low fat diet (n=23)*			*High fat diet (n=27)*			*Low fat diet (n=20)*		
	Before	***After***	***P***	***Before***	***After***	***P***	***Before***	***After***	***P***	***Before***	***After***	***P***
Weight (kg)	103.52 ± 25.50	97.43 ± 27.69	**<0.001**	96.76 ± 12.51	91.44 ± 13.25	**<0.001**	98.08 ± 17.25	91.40 ± 17.06	**<0.001**	95.61 ± 12.83	90.33 ± 12.51	**<0.001**
Waist (cm)	106.6 ± 17.35	100.03 ± 29.83	**<0.001**	100.70 ± 10.21	91.44 ± 13.25	**<0.001**	103.41 ± 12.05	96.96 ± 12.15	**<0.001**	104.26 ± 26.83	98.34 ± 24.61	**<0.001**
FFM (kg)	54.37 ± 6.79	53.12 ± 7.12	**0.003**	54.07 ± 5.17	52.80 ± 5.52	**0.009**	54.00 ± 5.77	52.59 ± 5.66	**<0.001**	53.59 ± 5.19	51.98 ± 4.88	**<0.001**
FM (kg)	49.15 ± 20.00	44.32 ± 21.64	**<0.001**	42.67 ± 9.08	38.64 ± 9.38	**<0.001**	44.10 ± 12.73	38.81 ± 12.68	**<0.001**	42.00 ± 11.13	38.35 ± 10.38	**<0.001**
Energy Intake (kcal/day)	1806.75 ± 505.24	1479.31 ± 173.90	**0.008**	2010.39 ± 564.04	1504.82 ± 162.79	**<0.001**	1958.91 ± 628.07	1446.71 ± 285.48	**<0.001**	2022.68 ± 451.94	1552.17 ± 266.11	**<0.001**
FFA (uM)	543.03 ± 128.96	557 ± 139.08	0.667	563.59 ± 159.75	463.89 ± 155.39	0.054	500.20 ± 119.78	534.89 ± 181.30	0.303	504.48 ± 171.54	466.90 ± 138.85	0.434
Glucose (uM)	5.17 ± 0.43	5.14 ± 0.38	0.591	5.23 ± 0.62	5.13 ± 1.23	0.216	5.19 ± 0.48	5.17 ± 0.53	0.774	5.31 ± 0.50	5.22 ± 0.47	0.314
Insulin (uU/ml)	11.37 ± 7.22	10.77 ± 7.12	0.858	10.44 ± 5.97	8.10 ± 7.31	**0.045**	7.88 ± 4.52	7.57 ± 5.12	0.595	10.50 ± 6.62	7.66 ± 4.98	**0.003**
Leptin (ng/ml)	38.81 ± 14.10	25.40 ±15.76	**<0.001**	33.70 ± 15.54	27.49 ± 13.18	**0.039**	32.87 ± 14.93	22.21 ± 12.44	**<0.001**	36.00 ±13.93	27.00 ± 14.57	**<0.001**
Cortisol nM)	192.68 ± 133.77	230.22 ± 148.57	0.088	213.34 ± 125.81	207.78 ± 128.69	0.756	201.50 ± 124.58	285.62 ± 156.07	**<0.001**	172.20 ± 84.19	259.61 ± 140.45	**<0.001**
IGF (ng/ml)	19.46 ± 7.82	22.09 ± 7.84	**0.024**	26.62 ± 14.13	27.95 ± 12.41	0.365	22.45 ± 6.41	23.42 ± 8.30	0.333	24.53 ± 6.43	25.51 ± 10.10	0.591
HOMA-IR	2.61 ± 1.70	2.47 ± 1.65	0.843	2.54 ± 1.77	1.98 ± 2.31	0.11	1.87 ± 1.23	1.81± 1.42	0.672	2.54 ± 1.70	1.82 ± 1.22	**0.005**

Values are means ± S.D. FFM, fat-free mass; FM, fat-mass; FFA, free fatty-acids; IGF, insulin-like growth factor; HOMA-IR, homeostasis model assessment -insulin resistance

Changes in adipose tissue gene expression

In this study ANOVA revealed a significant ($p<0.05$) time effect for all genes, independently of postprandial fat-oxidation rates or diet group (Figure 3.3.1).

SREBP-1c expression was unchanged in individual groups – although there was a trend in LXHF ($p=0.054$) and LXLF ($p=0.084$). HSL, MCAD and ATGL were significantly down regulated in HXLF and LXHF. apM1 expression dropped in HXLF. UCP2 was reduced in HXHF and LXHF with a trend in HXLF ($p=0.056$) (Table 3.3.2). ANOVA between subjects effects showed that HSL and MCAD were significantly different between the LX diet groups ($p<0.05$).

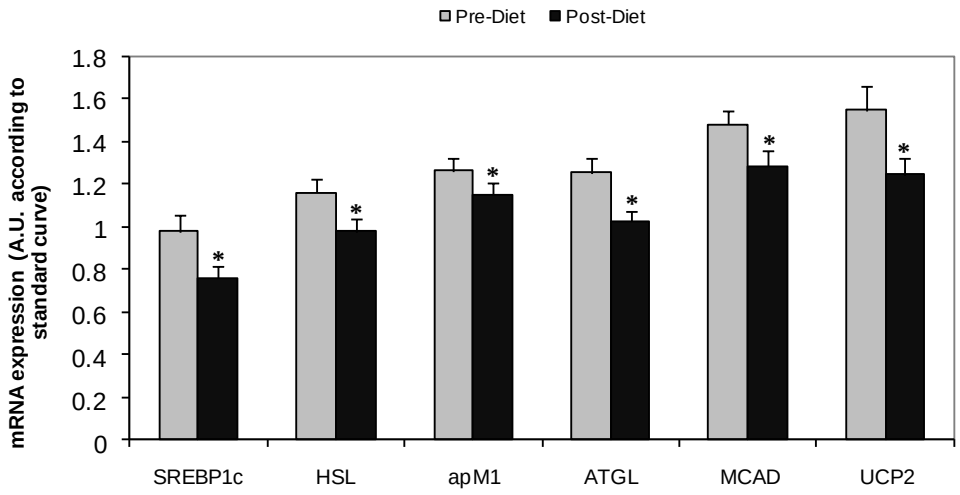


Figure 3.3.1 mRNA expression for metabolic genes, pre and post diet, independent of postprandial fat-oxidation rates or diet group. Units are A.U. according to the standard curve. Values are means ± S.D. SREBP-1c, sterol-regulatory-element-binding protein-1c; HSL, hormone sensitive lipase; apM1, adiponectin; ATGL, adipose tissue triglyceride lipase, MCAD; medium-chain acyl-coenzymeA dehydrogenase; UCP2, uncoupling protein-2. mRNA quantified using RT-qPCR.

Table 3.3.2 mRNA expression quantified using RT-qPCR. SREBP-1c, sterol-regulatory-element-binding protein-1c; HSL, hormone sensitive lipase; apM1, adiponectin; ATGL, adipose tissue triglyceride lipase; MCAD, medium-chain acyl-coenzymeA dehydrogenase; UCP-2, uncoupling protein-2.

	High postprandial fat oxidisers -1						**Low postprandial fat oxidisers - 2**					
	High fat diet (n=19)			*Low fat diet (n=23)*			*High fat diet (n=27)*			*Low fat diet (n=20)*		
	Before	***After***	***P***	***Before***	***After***	***P***	***Before***	***After***	***P***	***Before***	***After***	***P***
SREBP1c	0.698 ± 0.409	0.609 ± 0.431	0.199	1.077 ± 0.850	0.819 ± 0.625	0.109	0.899 ± 0.634	0.704 ± 0.541	0.054	1.251 ± 0.806	0.926 ± 0.550	0.084
HSL	1.037 ± 0.623	0.827 ± 0.495	0.085	1.072 ± 0.481	0.845 ± 0.323	**0.007**	1.061 ± 0.531	0.872 ± 0.436	**0.017**	1.314 ± 0.731	1.414 ± 0.912	0.540
apM1	1.139 ± 0.574	1.032 ± 0.405	0.337	1.320 ± 0.499	1.073 ± 0.353	**0.017**	1.205 ± 0.527	1.194 ± 0.512	0.910	1.404 ± 0.632	1.326 ± 0.585	0.593
ATGL	1.163 ± 0.626	0.937 ± 0.422	0.090	1.371 ± 0.610	1.036 ± 0.456	**0.006**	1.180 ± 0.680	0.973 ± 0.443	**0.046**	1.305 ± 0.562	1.194 ± 0.466	0.240
MCAD	1.486 ± 0.731	1.265 ± 0.740	0.176	1.398 ± 0.493	1.141 ± 0.411	**0.012**	1.420 ± 0.645	1.117 ± 0.425	**0.018**	1.659 ± 0.646	1.688 ± 1.037	0.888
UCP-2	1.513 ± 1.407	1.050 ± 0.690	**0.028**	1.374 ± 0.908	1.085 ± 0.560	0.056	1.556 ± 0.919	1.185 ± 0.757	**0.021**	1.790 ± 1.246	1.722 ± 0.876	0.713

Units are AU according to standard curve. Values are means ± S.D. SREBP-1c, sterol-regulatory-element-binding protein-1c; HSL, hormone sensitive lipase; apM1, adiponectin; ATGL, adipose tissue triglyceride lipase, MCAD; medium-chain acyl-coenzymeA dehydrogenase; UCP2, uncoupling protein-2. mRNA quantified using RT-qPCR.

General linear model analysis

General linear model analysis involved using an ANCOVA, where the effect of the covariate (in this case a phenotypic parameter or metabolite) is removed from the data-set, prior to performing an ANOVA test. This approach improves the power of the test by accounting for some of the variability inherent to human trials. After accounting for changes in phenotypic parameters or metabolites, further relationships with gene expression were observed. SREBP-1c showed a significant change in expression, when differences in responses to plasma FFA concentrations and HOMA-IR were allowed for. HSL expression was associated with plasma FFA whilst apM1 was found to be associated with HOMA-IR (Table 3.3.3).

Table 3.3.3 Covariate analysis for dietary/postprandial fat-oxidation groups. Values are A.U. showing mean differences between the groups.

	Phenotypic Variable	HXHF	HXLF	LXHF	LXLF	P
SREBP1c	Δ FFA	-0.067	-0.309	-0.161	-0.335	0.050
	Δ HOMA-IR	-0.202	-0.265	-0.234	-0.257	0.004
HSL	Δ FFA	-0.237	-0.176	-0.223	-0.112	0.023
apM1	Δ HOMA-IR	-0.151	-0.223	-0.042	-0.033	0.047

3.4 Discussion

Low-energy diets are commonly used to reduce BMI in obese and overweight individuals. We have assessed the interaction between fat oxidising capacity, composition of a low-energy diet, and change in relevant phenotypic characteristics in their effects on adipose tissue gene expression. The first, preliminary study revealed some phenotype – gene expression interactions, and we therefore refined our techniques to increase the sensitivity of our assays in the present study to see if differences in basic gene expression between the groups could be observed. The direction of change in gene expression in the first and second study was consistent in all transcripts.

The study was successful in significantly reducing body mass in both dietary groups. The context of fat oxidising capacity produced two distinct populations where gene expression was fundamentally altered, with significant reductions in four transcripts in HXLF and LXHF. Individually, neither fat oxidation status nor diet group were key determinants of gene expression. Analysis associated two of the phenotypic variables with the expression of three genes. The principal outcome of the study was the demonstration that fat oxidising capacity appears to have a dietary fat content dependent impact on the regulation of gene expression in adipose tissue during low-energy diets.

Individuals with high or low fat oxidising capacity may have developed this phenotype through long-term dietary preferences, inter-genomic variation – or more likely a combination of these factors (Keskitalo *et al.*, 2007; den Hoed *et al.*, 2008).

SREBP-1c is the master regulator of lipogenic genes, and its suppression in this study is small but significant ($P<0.05$) in the cohort as a whole. GLM analysis correlated SREBP-1c with FFA and HOMA-IR. Insulin induces skeletal muscle SREBP-1c in humans (Boonsong *et al.*, 2007) and fasting suppresses it in rat adipose tissue (Gosmain *et al.*, 2005). SREBP-1c expression also decreased ~30% in the adipose tissue of obese subjects post-diet (Ribot *et al.*, 2001). Since fasting plasma insulin was unchanged, they proposed that reduced fat mass was responsible. However, Kolehmainen et al also found no link between insulin sensitivity and SREBP-1c expression in obese subjects (Kolehmainen *et al.*, 2001). Therefore, it may be the case that in obese insulin resistant individuals, FFAs act as the primary mechanism of regulation via PPARs (Pegorier *et al.*, 2004; Jump *et al.*, 2005a), whereas insulin plays a role when its signalling pathways are conserved.

NUGENOB studies preceding this one – albeit smaller in size, with different cohorts of subjects and not focussed on fat oxidation - had revealed a pronounced decrease in the expression of HSL and ATGL (Viguerie *et al.*, 2005; Jocken *et al.*, 2007), and as a result they had suggested that there might be a co-regulatory pathway. Our data strongly confirm this association, seen in the group-specific suppression of the two lipases, with a trend in HXHF, and significant changes in HXLF

and LXHF. HSL and ATGL expression are suppressed by insulin, and it is possible that subtle improvements in insulin signalling were responsible for this (Jocken *et al.*, 2007). MCAD is required for the first step in the β-oxidation of fatty acids; and its down regulation in HXLF is probably attributable to the decreased lipid handling requirements by the cell following energy restriction. MCAD expression in LXHF was similar to the other groups prior to the diet, but was significantly reduced afterwards. This is hard to reconcile with the requirements of the cell, and it could be that the phenotype is ill-adapted to using the dietary fat, or perhaps part of a wider PPARγ Coactivator-1a (PGC1a) - Estrogen Related Receptor (ERR) mediated response to underfeeding, which subsequently affects MCAD as the cell adapts to reduced energy intake, rather than fuel availability (Kamei *et al.*, 2003).

The regulation of UCP2 expression in adipose tissue is less clear. Induction occurs with short term fasting in both lean and obese subjects, but is not affected by acute insulin infusion (Millet *et al.*, 1997). UCP2 has been shown to be suppressed in previous weight loss studies (Viguerie *et al.*, 2005). However, in our study, it was more specifically the high fat diet groups which were affected. The suppression of UCP2 during this long term intervention could be part of the mechanism by which lower REE is observed after weight loss. This is thought to be an adaptation of the human body in response to starvation (i.e. evolutionarily advantageous in times of famine), and, as Rosenbaum et al have described, this is a persistent phenotype, which may explain the difficulty in maintaining post-dietary weight loss (Rosenbaum *et al.*, 2008). Although FFAs are responsible for PPAR activation, and PPARs are the main activator of UCP induction, weight loss appears to be the determining factor.

Adiponectin mRNA and protein expression are lower in obese individuals (Arita *et al.*, 1999), contrary to what might be expected, since adiponectin is secreted exclusively from adipocytes (Scherer *et al.*, 1995). However in the HXLF group, adiponectin was also significantly lower after the diet – this is unusual since adiponectin expression typically increases with weight loss. GLM analysis associated adiponectin expression with HOMA-IR in all groups; this is in accordance with the

observation that decreased circulating adiponectin is associated with the development of insulin resistance (Davis & Scherer, 2008).

The vast majority of significant changes in gene expression are seen in the HXLF and LXHF groups, this is most likely due to a greater need for the cell to adapt to the changes in lipid availability, transport and oxidation, because of the initial phenotype being mismatched with the subsequent dietary fat compositions. In the case of HXLF, a reduced requirement for postprandial fat oxidation during the low fat diet, is reflected in the downregulation of gene expression. This may support a hypothesis that the HX phenotype results from long-term habitual high fat consumption. LXHF may have been unable to utilise the extra dietary fat, and have therefore reduced energy expenditure, alongside candidate gene expression. Although fat oxidation groups did reveal important differences in the changes in gene expression, as described above, these were part of an adaptation to the diet. Changes in gene expression were not a key determinant of BMI reduction during the low-energy diet; with reduced energy intake exerting fundamental control of metabolic gene expression.

The differences observed in the transcriptome may have been stabilised over many years by their exposure to particular diets. Transcriptional pathway analysis and integration with metabolomic data would be the next step in providing a comprehensive assessment of which factors in metabolic gene expression are responsible for fat oxidation rates, and subsequent weight loss during energy restriction. Future studies should also include a post-diet postprandial fat oxidation test to assess whether there are any changes in fat oxidising capacity during these diets.

Acknowledgments

We would like to thank the participants of this study and also the NUGENOB consortium members for their cooperation (see http://www.nugenob.com/). The NUGENOB study was funded by the EU under the 5th Framework Programme (Grant No QLK1-CT-2000-00618), and the present study was funded by Nestlé and supported by FRAME.

Chapter 4

Transcriptional Profiling of Adipose Tissue in Obese Subjects with High- or Low-Postprandial Fat Oxidising Capacity

4.0 Transcriptional Profiling of Adipose Tissue in Obese Subjects with High- or Low-Postprandial Fat Oxidising Capacity

4.1 Introduction

Adipose tissue, originally considered to be metabolically inert, is now recognised as an important regulator of energy balance. It exerts its actions via adipokines and also through its energy storage and mobilisation potential (Sethi & Vidal-Puig, 2007). The ability to store or utilise dietary fat varies between individuals, but there have only been cursory investigations into how gene expression in adipose tissue is affected by whole-body fat oxidation rates (and vice-versa). Rising obesity rates are a concern for industrialised nations, and challenge to healthcare provision. In obese individuals, adipose tissue depots increase in size leading to changes in; insulin sensitivity, osteoarthritis, cardiovascular function, and predisposition to various cancers (Calle *et al.*, 2003; Kahn *et al.*, 2006; Van Gaal *et al.*, 2006; Teichtahl *et al.*, 2008).

Obesity is the result of a long-term, positive energy balance - caused by high energy intake and low energy expenditure. Studies have shown that energy expenditure appears to differ depending on the dietary fuel substrate consumed; a factor which may contribute to weight gain. Increased dietary carbohydrate intake leads to a rapid increase in carbohydrate oxidation rates (Blaak & Saris, 1996), with insulin dependent downregulation of PDK4 (Lee *et al.*, 2004; Tsintzas *et al.*, 2007). Increasing dietary fat does not produce the same increase in fat-oxidation rates, with a slower and more blunted adaptation (Schrauwen *et al.*, 1997) (Schutz *et al.*, 1989b; Thomas *et al.*, 1992).

The relationship between fat oxidation and obesity is complicated. There is evidence that postprandial fat oxidation rates are lower in obese individuals (Blaak *et al.*, 2006), and that fat oxidation rates in individuals susceptible to becoming obese may be lower; or alternatively as Astrup, Buemann *et al* have suggested, obesity could be viewed as an adaptation to a low fat-oxidation rate; whereby through becoming

obese, and consequently insulin resistant, this reduces carbohydrate oxidation and promotes higher-rates of fat oxidation (Astrup *et al.*, 1994). It should also be noted that there is considerable variation in fat oxidation rates between individuals in obese populations, with a range that can also be observed in lean subjects. It remains unresolved as to how different postprandial fat oxidation rates are maintained. Understanding how fat oxidation in obese subjects is related to gene expression in adipose tissue, could provide more effective, dietary strategies, personalised treatment options and improved clinical outcomes for obese patients.

In this investigation, the transcriptional profiles of adipose tissue in high- and low- postprandial-fat oxidising obese subjects were compared through microarray analysis. The main focus of this investigation has been to look at gene expression more generally, without bias, to see whether genes can be discriminated on the basis of postprandial fat oxidising capacity. This study will also highlight areas where further research might be necessitated. Secondly, the genomic contribution to obesity, assessed via genome-wide association studies (GWAS) has yielded interesting targets such as FTO (Scuteri *et al.*, 2007) and MC4R (Loos *et al.*, 2008) which are most likely involved in the energy intake, but to-date fat oxidation has only been linked to a small number of genes (Hofker & Wijmenga, 2009). The present study examines whether expression Quantitative Trait Loci (eQTLs) contribute to the high- and low-postprandial fat oxidising phenotypes, and to investigate any potential genetic component to changes observed in the transcriptome.

4.2 Materials and methods

Subjects and study protocol

771 Caucasian Europeans (579 pre-menopausal women) all of whom were obese (age 20-50 yr, BMI ≥ 30 kg/m^2) , along with 119 lean control subjects, participated in the NUGENOB study, funded by the European Union, which is described by Petersen et al. (Petersen *et al.*, 2005). The subjects for the present study (all female; non-diabetic) were selected from this population, on the basis of their

respiratory quotient (RQ) after a high-fat test meal. The test meal consisted of double cream (40g fat/100g, with butter used to standardise the fat percentage in 3 centres). Subjects were required to consume the meal within 10 minutes. The fat oxidation data was used to rank subjects, with the top and bottom 100 subjects referred to as the high (HX) and low (LX) –postprandial fat-oxidising groups, respectively. Selection was made at random from these categories to form two distinct and non-overlapping groups; HX, n=17; LX, n=18 (Figure 4.2.1). All subjects were matched for HOMA-IR, body mass and composition.

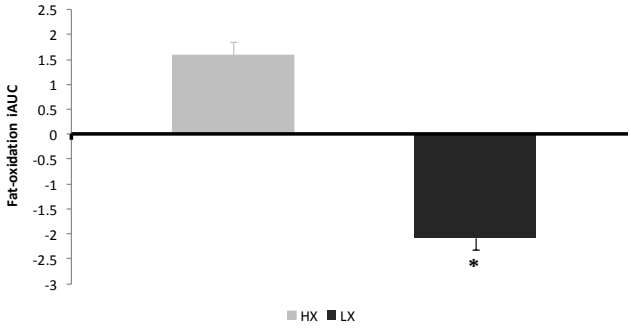


Figure 4.2.1 Postprandial fat oxidation rates (iAUC) for HX and LX groups, relative to lean control subjects. Bars represent means ± S.E.M. *P<0.001.

Biochemical analyses

Venous blood samples were drawn after an overnight fast of 12 h, following a 3-day period when subjects had been instructed to avoid excessive physical activity or alcohol consumption. Subjects rested in the supine position for 15 min prior to the procedure. Blood metabolites were assayed using techniques previously described.(Petersen *et al.*, 2005; Viguerie *et al.*, 2005)

RNA isolation

Adipose tissue biopsies were taken from subcutaneous abdominal fat (~1 g). The adipose tissue specimens were stored in RNA Later solution, at -80°C. RNA was extracted using the RNeasy total RNA Mini kit (Qiagen, Courtaboeuf, France) at the

INSERM laboratory in Toulouse. The quality of the RNA was assayed using a 2100 Bioanalyser (Agilent Technologies, Massy, France).

Gene expression

1µg of RNA was subjected to ribosomal RNA reduction using the RiboMinus™ Transcriptome Isolation kit (Human/Mouse) (Invitrogen) along with the Magna Sep™ magnetic particle separator (Invitrogen), prior to cDNA synthesis. After this, the Affymetrix GeneChip® Whole Transcript (WT) Sense Target Sense Target labeling assay as described in the protocol. The ssDNA was then hybridised to Affymetrix GeneChip® Human Exon 1.0ST arrays, and quality control checks were performed using Affymetrix Expression Console software.

mRNA quantification by Real Time – Quantitative PCR (RT-qPCR)

cDNA was synthesized from 1µg of RNA (after DNase treatment), using Superscript III Supermix for qRT-PCR (Invitrogen) in accordance with the protocol; the reaction was carried out on a GeneAmp PCR System 9700. Probes and primers for TaqMan® RT-qPCR were designed using Primer Express V2.0 software (Applied Biosystems, UK) and manufactured by MWG (Germany). The reaction was carried out in a 96-well Micro-amp plate, utilising both an ABI Prism 7700 Sequence Detection System and an ABI StepOne Plus (Applied Biosystems, UK). Probes and primers were used at a final concentration of 10µM in a reaction volume of 25µL, containing 5µL of cDNA. Assays were performed in triplicate with the average threshold cycle (Ct) values normalised using either RPLP0 or 18S. The Ct values for RPLP0 and 18S did not change significantly between time-points, nor was there any bias between machines. The slope of the standard curve was maintained between -3.1 and -3.6.

Genotyping

Illumina Sentrix® HumanHap300 BeadChips (Illumina, San Diego, CA) were used for high-throughput SNP genotyping, which was carried out at Integragen (Evry,

France) Baseline blood samples were used for the extraction of DNA from buffy coat, carried out at the Steno Diabetes Centre in Copenhagen.

Data analysis

Affymetrix HuEx 1.0ST arrays were analysed using GeneSpring GX11. Normalisation was performed using the RMA16 summarization algorithm. Transcripts with a p-value ≤0.05, after Benjamini- Hochberg multiple testing correction and a fold-change ≥1.3 were considered both statistically and biologically significant. A Euclidean distance metric was used to define co-expression of transcripts. Alternatively spliced transcripts were detected by DABG (p≤0.05), application of a splicing ANOVA (p≤0.05), and a splicing index threshold of 0.5 (≈1.4 fold difference). PLINK (Purcell *et al.*, 2007) was used for the analysis of HumanHap300 BeadChip SNP data, using the options; missing rate per person (mind), 0.05; missing rate per SNP (geno), 0.05; minor allele frequency (maf) 0.02, Hardy-Weinberg equilibrium cut-off P=0.001, to determine whether any SNPs were associated with the postprandial fat oxidizing capacity, or gene expression. 817 genes with a fold-change ≥1.2 were used for eQTL analysis. The genomic inflation factor was used to correct for batch effects and to assess genome wide significance, the threshold of which was 10^{-8}. Geographical structures within the data (Figure 4.3.5) were accounted for, by allocating centre numbers as a covariate. Linkage disequilibrium (LD) blocks were defined using the Gabriel confidence rule in Haploview (Barrett *et al.*, 2005) and the CEU population in HapMap. Ingenuity Pathway Analysis v11 (Ingenuity Systems, CA) was used to find relationships between differentially expressed genes. GLM covariate analysis of metabolite-gene expression relationships was performed using SPSS V. 15 (SPSS, Chicago, IL).

4.3 Results

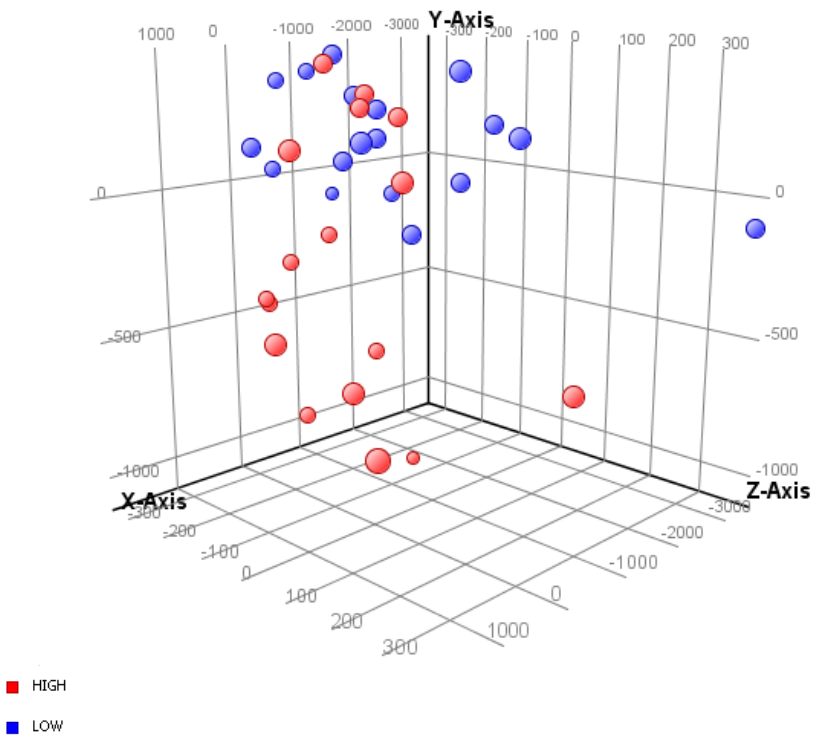


Figure 4.3.1 Principal component analysis (PCA) plot showing the distribution of subjects along 3 eigenvectors.

Gene expression

Principal component analysis (PCA) of global gene expression profiles revealed two distinct populations based on postprandial fat oxidation group (Figure 4.3.1), with eigenvalues of X =56.95, Y=23.96, Z=10.87. Out of a total 15715 transcripts with detectable level of expression, 179 ($p<0.05$) and 112 ($p<0.001$) were expressed with a fold change ≥1.3. (Appendix I)

RT-QPCR was used to validate changes in mRNA expression –relative to HX – for; FASN (1.47), GLUT4 (1.59), RXRA (1.44) & SREBP1c (2.25) (Figure 4.3.2). One other transcript – ADRA2A could not be validated due to a large S.E.M, although directional concordance was observed. Of the 179 transcripts exported for analysis in IPA, any connection between two or more entities was retained (self-regulatory motifs were therefore not included) (Figure 4.3.3).

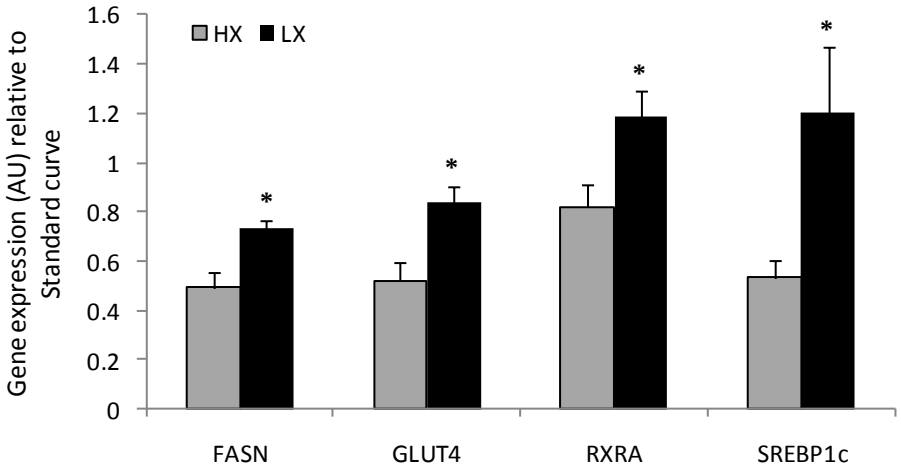


Figure 4.3.2 mRNA expression for FASN, GLUT4, RXRA and SREBP1c. Values are mean ± S.E. *P<0.05.

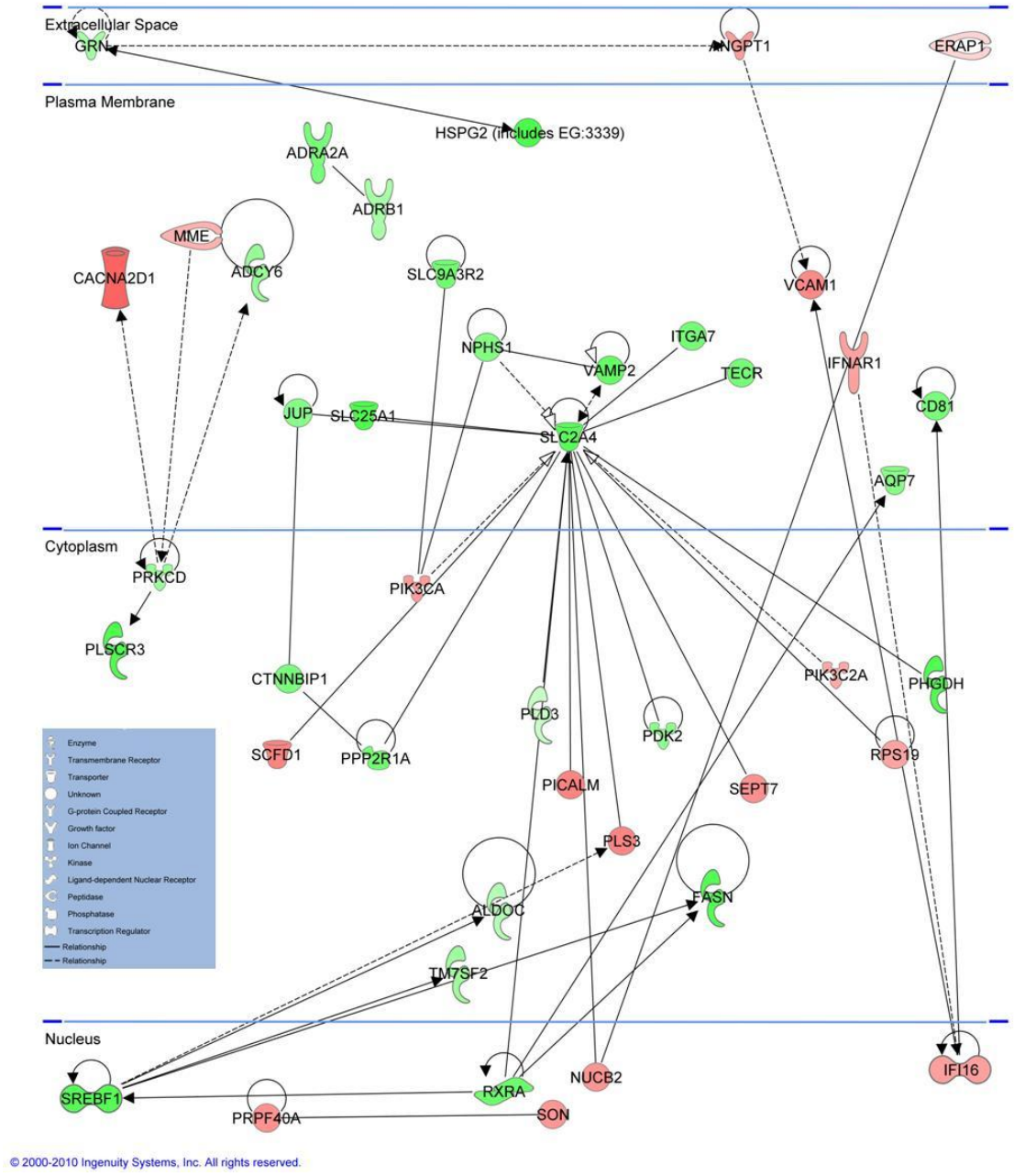


Figure 4.3.3 Pathway analysis using IPA v11. Connect rule; using Human, Rat and Mouse data. Solid lines are direct relationships, dashed lines are indirect relationships. mRNA expression is shown using green and red. Green indicates higher expression in LX. All transcripts shown had a fold-change >1.3, and $P<0.05$.

Table 4.3.1 Co-expression of key metabolic transcripts. Values calculated using an Euclidean distance metric.

Gene symbol	Genbank Ac. #	Similarity
FASN	**U29344**	**1.000**
SLC2A4|GLUT4	BC034387	0.952
SLC2A4|GLUT4	**BC034387**	**1.000**
FASN	U29344	0.952
ALDOC	**BC003613**	**1.000**
OR3A2|OR3A3	BC108921	0.924
ADRB1	J03019	0.916
SLC7A10	AB037670	0.904
OR4D1		0.902
PNPLA2|ATGL	**BC017280**	**1.000**
RNPEPL1	BC082975	0.977
PLSCR3	AK124006	0.977
ZFP36L2	BC005010	0.976
LRP3	AB009462	0.974
PHGDH	AK093306	0.973
HSPG2	M85289	0.969
KIAA0515	BC012289	0.956
SREBF1	**BC057388**	**1.000**
SLC25A1	L75823	0.922
FXYD1|FXYD7	AK289811	0.916
SLC2A4	BC034387	0.887
RXRA	**BC063827**	**1.000**
CRIP2	AK091845	0.987
VAMP2	AK021522	0.955
KIAA0195	BC042942	0.951

Alternative Splicing

402 transcripts showed alternative splicing including; SREBF1, ADRB1, ATGL (PNPLA2) and PNPLA3 (Figure 4.3.4). RT-qPCR validation of SREBF1 showed that there was no difference between the abundance of the -1a splice variant between groups, but that -1c was significantly more abundant in LX than in HX($P<0.05$).

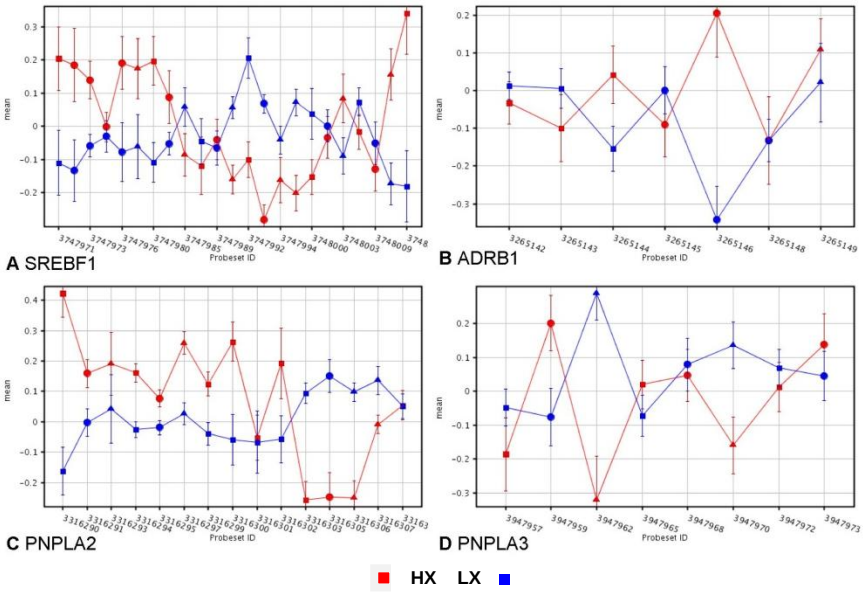


Figure 4.3.4 Exon specific (each shape is one exon) mRNA expression for; A, SREBF1; B, ADRB1; C, PNPLA2 (ATGL); D, PNPLA3 (ADPN).

eQTL assessment

Out of the 817 transcripts assessed for eQTL status, 28 (35 SNPs) were found to have statistically significant values after genomic control was applied. 4 SNPs were located on the same chromosome - although none were cis-acting (within 1Mb of the transcript). 14 trans-acting SNPs lay within the coding regions of other transcripts or at splice junctions (Appendix I). For example, rs861826, which was associated with the expression of Early Endosome Antigen-1 (EEA1) was located within the LD block for both a non-synonymous-coding and Splice-site SNP (Appendix I).

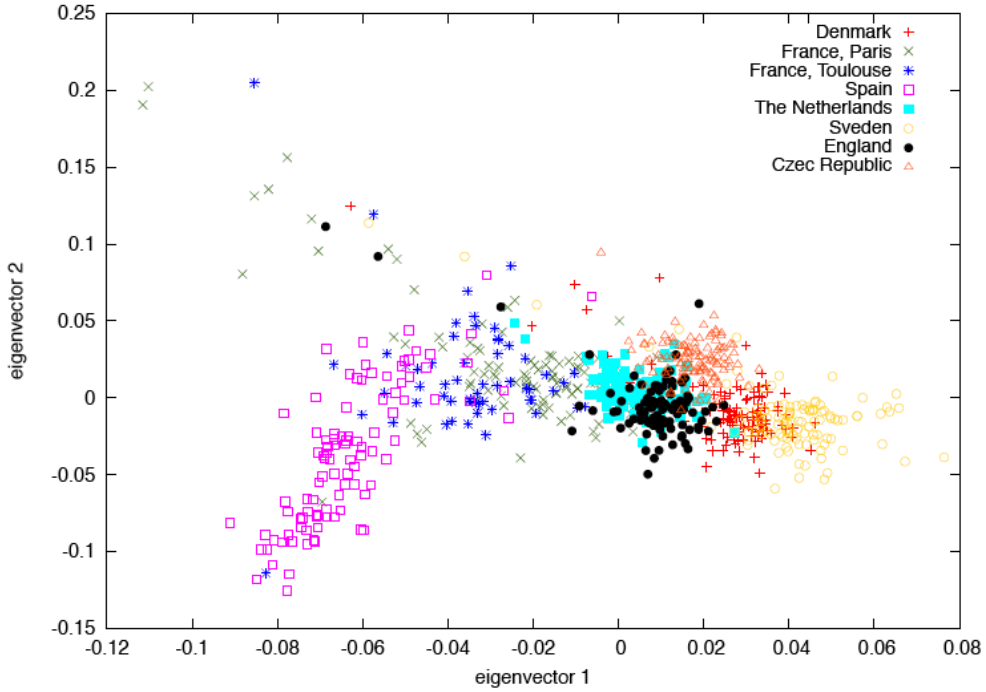


Figure 4.3.5 PCA plot showing the geographic distribution of SNP data for centres involved in the NUGENOB study. Image courtesy of Tune Pers, CBS, Denmark.

4.4 Discussion

These data suggest a central role for adipose tissue GLUT4, FASN, AQP7, and the transcriptional regulators SREBP1c and RXR in the maintenance of postprandial fat-oxidation phenotypes. Low postprandial fat-oxidisers may also have improved lipid storage and mobilisation, which results from their decreased use of FFAs as a fuel substrate.

GLUT4 is the insulin regulated transporter of glucose, and its higher expression in low fat oxidising subjects, along with other associated entities (Figure 4.3.3) is indicative of an increased potential for glucose uptake into adipocytes. However, GLUT4 is not differentially expressed in isolation. The transcriptional regulators, RXRA and SREBF1 are both upregulated in the LX group, along with their targets; FASN, ATGL, AQP7 and ALDOC. RXRA regulates the expression of SREBP1c (Kamei *et al.*, 2008), and both of these transcription factors are responsible for the lipogenic program in adipose tissue (Pegorier *et al.*, 2004). The contribution of

de novo lipogenesis to lipid storage remains controversial (Chascione *et al.*, 1987; Diraison *et al.*, 2003), but expression of FASN is tightly correlated with lipid synthesis rates in adipose tissue (Wang *et al.*, 2004). These results indicate that low postprandial fat-oxidisers, have better control over the storage and mobilisation of dietary lipids. If the ability to control TAG lipolysis more effectively is the outcome of these changes in gene expression, then increased AQP7 would allow the export of glycerol to be used as a fuel substrate (Maeda *et al.*, 2009). The purpose of the increased GLUT4 expression could be to import glucose to regenerate glycerol, required for the storage of FFAs as TAGs as glycerol released during lipolysis cannot be reutilised in TG synthesis. GLUT4 expression was highly co-expressed with FASN (Table 4.3.1); almost certainly induced via RXRA.

The cause of these differences in gene expression could be that FFA metabolism in LX muscle and other peripheral tissues is impaired in some way, which therefore forces WAT to store the lipids, and it does this by altering metabolic gene expression. This would concur with the idea that high fat oxidisers rapidly metabolise dietary fat after consumption. However, subjects in this study were matched for HOMA-IR, and were not insulin resistant; as a result it cannot be suggested, in this study, that any impairment in fat oxidation is the result of insulin resistance in muscle.

Other transcripts which were differentially expressed include those familiar to vascular endothelial cells; VCAM1, ANGPT1, IFNAR1 and IFI16, which may be consequential to the vascular structure of WAT, perhaps by reducing blood flow to the tissue, and subsequently the availability of nutrients – which could contribute to the changes observed in lipolytic genes. However these transcripts are also involved in inflammatory responses and may be a result of differences in inflammation between the two groups, a factor which is linked to the development of insulin resistance (Ferrante, 2007; Ingelsson *et al.*, 2008).

The large number of alternatively spliced transcripts reveals the potential importance of splicing in determining the difference between the postprandial fat oxidising phenotypes. Alternative splicing events in SREBF1 can be ascribed to differences in the lipid handling requirements of the cell. The higher levels of SREBP-

1c mRNA observed in LX ($p<0.05$) would be anticipated in response to increased storage of FFAs (Osborne, 2000) . Varying differences in the abundance of SREBP1c & -1a have been reported depending on the organism and tissue type, with -1c being 3 times more abundant than -1a in adipose tissue (Shimomura *et al.*, 1997). In this study, the expression of -1a was approximately equal in the LX and HX groups (Appendix I). Differences in the expression of SREBF1 can therefore be attributed to -1c.

PNPLA2 (ATGL) and PNPLA3 (ADPN) were both alternatively spliced between the two groups, with splicing events occurring in the Acyl_Trfase/lysoPlipase superfamily domains, which could affect catalytic efficiency (Figure 4.3.4)(Jenkins *et al.*, 2004; Kim *et al.*, 2006b). ATGL and ADPN are both lipases which have been linked to energy balance (Liu *et al.*, 2004). The picture regarding these lipases is complicated with experimental data showing that ATGL is down regulated by insulin in 3T3-L1 adipocytes (Kim *et al.*, 2006a), but also decreased in the obese insulin resistant state (Jocken *et al.*, 2007). Conversely, evidence suggests that ADPN is upregulated in (insulin sensitive) obesity, and that it may have also have a lipogenic role (Johansson *et al.*, 2006). A reduction in lipolytic/lipogenic capacity, resulting from alternative splicing, could significantly reduce energy expenditure during weight loss diets, and therefore success during such programmes.

Alternative splicing and increased ADRB1 expression (Figures 4.3.3 & 4.3.4) in LX could point towards an altered lipolytic response when stimulated by catecholamines. Decreased expression of β_2-adrenoceptors has already been implicated in the development of obesity (Haffner *et al.*, 1993; Reynisdottir *et al.*, 1994) with some studies associating ADRB1 polymorphisms with metabolic dysregulation (Lima *et al.*, 2007).

Since Brown Adipose Tissue (BAT), which has a high metabolic rate, is now recognised as being present in adult adipose tissue masses (Nedergaard *et al.*, 2007), we looked for the BAT specific marker PRDM16 (Seale *et al.*, 2008) – which was not expressed, although PRDM16 has been linked to BAT depots rather than diffuse

brown adipocytes, which probably have a different developmental origin (Ravussin & Kozak, 2009).

Integration of SNP data revealed several associations with gene expression, but none where the SNPs lay within the coding genes, or the surrounding LD block. Some of the associated SNPs lay within the coding regions of other genes, and their involvement may show that they are involved in the same pathways or are interacting through epistasis. The current understanding of the genome restricts interpretation of this data. 5 SNPs were associated with the expression of genes involved in GLUT4 translocation (Appendix I), although the significance of this finding is unclear due to the limited knowledge of epistatic and pathway interactions. Another study from the NUGENOB project examined 42 SNPs in isolation using a larger number of subjects and associated IL-6 with postprandial fat oxidation rates, although it was reported with the caveat that IL6 is also released from working muscle – which makes interpretation of the role in adipose difficult. They also found no association with the FTO gene, which was interpreted as a lack of influence of FTO on fat oxidation. (Corpeleijn *et al.*, 2009). In the present study neither IL-6 or FTO were associated with eQTLs.

Co-expression analysis of several key genes clarifies the effects of transcriptional regulation, with FASN and GLUT4 having very closely linked profiles (Table 4.3.1), which is likely due to their shared modes of induction via RXRA:LXR and SREBP1c (Joseph *et al.*, 2002b; Dalen *et al.*, 2003). SREBF1 was also coexpressed with GLUT4; therefore it is possible that this synergy between two transcription factors (RXRA & SREBF1) drove the higher expression in LX.

Covariate analysis (data not shown) of RXRA expression also showed an interaction with fasting blood glucose levels p=0.041. The physiological significance of this is unclear, although as previously discussed; RXRA is involved in the regulation of GLUT4 expression, although GLUT4 expression itself was not linked with fasting blood glucose (FBG) concentrations in this study. ALDOC (P=0.038) & ATGL (P<0.036) also presented an interaction with FBG. HSL, which is often co-expressed with ATGL, showed no relationship with FBG. This difference in responsiveness

between ATGL and HSL demonstrates the basal role that ATGL plays in lipolysis in adipose tissue and also its place in the glucose-fatty-acid cycle (Langin *et al.*, 2005).

This study has outlined differences in the transcriptional profiles of obese subjects with different postprandial fat-oxidising capacities. Adaptations to low postprandial fat-oxidation involve the use of glucose for storage and mobilisation of lipids, principally under the control of RXRA and SREBP1c. Potential methods of increasing fat oxidation such as upregulating UCPs or modifying appetite using agonists for MC4R, will need to take account of differences in lipid mobilisation, and whether these differences in postprandial fat-oxidation have an epigenetic or genomic cause.

Subsequent studies in this area should also attempt to examine the expression of skeletal muscle metabolic gene expression in relation to the observed changes in adipose tissue biology, to see whether impairment of postprandial fat oxidation is the cause of alterations in adipose tissue gene expression, and also whether decreased lipolysis might lead to an increase in IMTG storage – potentially a contributing factor in peripheral insulin resistance. The sequencing of further genomes and methylomic analysis offer another avenue to investigate the impact of, less common genetic variants with large influences on energy balance, and the role of epigenetics in the development of these phenotypes.

Chapter 5

Effects of high-glucose treatment on metabolic gene expression in Human primary myotubes

5.0 Effects of high-glucose treatment on metabolic gene expression in Human primary myotubes

5.1 Introduction

Type 2 Diabetes Mellitus (T2DM) is characterised by high fasting blood glucose concentrations. This situation is termed hyperglycaemia, and has a number of serious negative physiological effects including; retinopathy, nephropathy and neuropathy. The development of hyperglycaemia (defined by the WHO as fasting blood glucose concentrations ≥7.0mmol/l) is usually a consequence of insulin resistance (WHO, 2006a). Insulin resistance occurs when the release of normal levels of insulin from β-cells in the pancreas fails to promote an adequate increase in glucose uptake from insulin-responsive cells. Since skeletal muscle is the main site of insulin stimulated glucose-disposal, the effects of skeletal muscle insulin resistance on whole-body glucose homeostasis can be severe.

In obesity and diabetes (obesity often preceding the development of diabetes); ectopic fat distribution is thought to contribute to the development of systemic-insulin resistance. Ectopic fat distribution in skeletal muscle is via the accumulation of intramyocellular triglycerides (IMTG) in response to nutrient oversupply – commonly increased fat intake (Ravussin & Smith, 2002). IMTG content has been linked to the development of insulin resistance, but whether it is a causal relationship remains unclear, especially since trained athletes have high IMTG content in their skeletal muscle (van Loon & Goodpaster, 2006). Although IMTG accumulation is associated with chronically elevated blood lipid profiles; hyperglycaemia may contribute to the increased production of IMTG by promoting *de novo* lipogenesis (DNL) in skeletal muscle (Bandyopadhyay *et al.*, 2006). Glucose induced insulin resistance has been demonstrated in several studies (Vuorinen-Markkola *et al.*, 1992; Del Prato *et al.*, 1994; Boden *et al.*, 1996), and synthesis of IMTG in skeletal muscle, may link these observations. The transcriptional activation of lipogenic proteins, and therefore DNL, in skeletal muscle thought to be under the control of sterol-regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding-

protein (ChREBP), both having been established as the transcriptional activators of a number of lipogenic enzymes, most notably acetyl co-A carboxylase (ACC) and fatty acid synthase (FAS) (Dentin *et al.*, 2005b). LXR may contribute to the activation of lipogenic gene expression, as has been demonstrated in primary myotubes using by modulating LXR activity (Kase *et al.*, 2007), although more recent work has shown that ChREBP alone, and not LXR, induce glucose regulated genes in mouse liver (Denechaud *et al.*, 2008). This action has not yet been established in skeletal muscle.

The aims of the present study were; to establish how the expression of metabolic genes involved in carbohydrate oxidation and *de novo* lipogenesis, might be altered in response to hyperglycaemia – without the influence of insulin signalling. It was anticipated that lipogenic gene expression such as FAS and ACC would be upregulated, under the control of ChREBP. This study was carried out using high-glucose treated human primary myotubes. 45 metabolic genes including; transcriptional activators, cells signalling proteins, substrate transporters, lipogenic enzymes, and proteins catalysing glucose and fat oxidation were observed, after incubation in either normal (5mM control) or high-(18mM) glucose media for 4 and 24 hour periods.

5.2 Materials and methods

Muscle biopsy

Young (20-40 years), male non-diabetic subjects were fasted overnight, and rested for 30 minutes prior to muscle biopsy. Muscle biopsies were obtained from the *vastus lateralis* under local anaesthetic using percutaneous Bergstrom needles.

Cell culture

Muscle biopsy tissue was digested and the satellite cells prepared as described (2.3). Cells were plated into 14 gelatine-coated T25 flasks (6 control; 8 treated), and grown until they became fully differentiated myotubes. Primary myotubes

were then treated with media containing either 5mM (Control) or 18mM (High) glucose, for 4 hours and 24 hours, before RNA extraction.

RNA isolation

Total RNA was isolated from muscle biopsies using Trizol (Invitrogen), and further purified using RNeasy RNA clean up columns (Qiagen). RNA concentration was determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Delaware, USA).

Gene expression

ABI StepOne+ RT-qPCR

1 µg of RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen), and quantified using an ABI StepOne Plus (Applied Biosystems, UK). Probes and primers were used at a final concentration of 10µM in a reaction volume of 25µL, containing 5µL of cDNA. Assays were performed in triplicate with the average threshold cycle (Ct) values normalised using RPLP0. The Ct values for 18S did not change significantly between time-points. The slope of the standard curve was maintained between -3.1 and -3.6.

Taqman® Low-Density Array

Taqman® Low-Density Custom Array using Micro Fluidic cards (ABI Applied Biosystems, UK) was used for the quantification of expression of 48 key metabolic genes in human skeletal muscle. Each card allowed for 8 samples to be run in parallel against 48 Taqman gene expression assay targets that were pre-loaded into each of the wells on the card. Briefly, 50 µl of Taqman Universal PCR master mix (2x) (ABI Applied Biosystems, UK) was added to 250 ng RNA equivalent of cDNA into an eppendorf RNAse free tube. RNAse free water was then added to make the total volume of the reaction mixture up to 100 µl. The reaction mixture was then vortexed, centrifuged and loaded into one of the fill reservoirs of the Micro Fluidic card. The cards were then centrifuged (MULTIFUGE 3 S-R, Heraeus) and run on a 7900HT Fast

Real-Time PCR System (ABI Applied Biosystems, UK). The expression of hydroxymethylbilane synthase (HMBS) was used to normalise the data to minimize variations in the expression of individual housekeeping genes. Gene expression was measured using the $2^{-\Delta\Delta}$Ct method

Statistical Analysis

All data were analysed with 2-way ANOVA using SPSS v.16 (Chicago, IL). Results are presented as fold-change ± S.E.M. Statistical significance was accepted at $P<0.05$.

5.3 Results

RT-QPCR

A preliminary study, using the same protocol as described above – was used to determine whether there were changes in PDK4 expression, prior to using the low-density custom array. Measuring only PDK4 expression using StepOne RT-qPCR, this study showed a significant (P<0.05) suppression of mRNA abundance at 4 hours (-2.19-fold) in the high-glucose treated cells relative to the control. This was followed by a sharp and significant 7.66-fold increase at 24 hours – which was also significantly higher than the control (Figure 5.3.1).

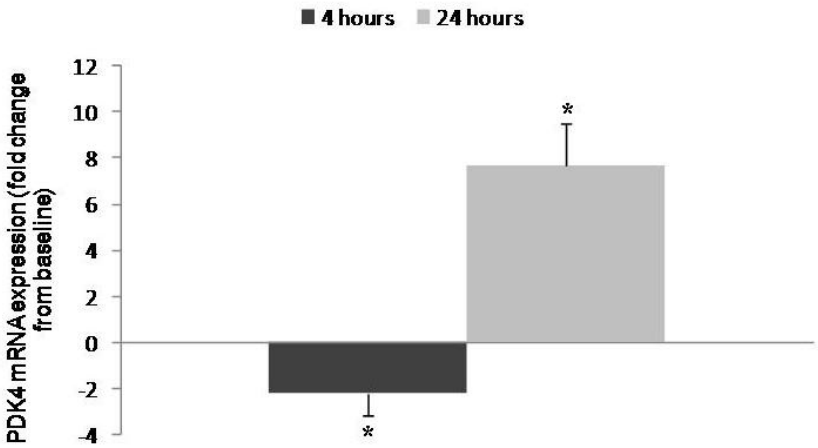


Figure 5.3.1 PDK4 mRNA expression. Values are fold-change ± S.E.M. n=6. *P<0.05 from baseline. PDK4, pyruvate, dehydrogenase kinase.

Taqman Low-Density Array

Table 5.3.1 Changes in metabolic gene expression, in response to 4 and 24 hours high-glucose treatment.

	Treatment		Time	
	N4 v H4	N24 v H24	N4 v N24	H4 v H24
PDP2			2.78	
ADFP				2.75
FASN				2.59
IRS2				1.72
CHREBP		+		+
PDK4	-1.58			2.32
DGKD		2.12		

Significant (P<0.05) fold-changes in gene expression between i) treatment, relative to control; and ii) time, relative to 4 hours. PDP2, pyruvate dehydrogenase phosphatase 2; ADFP, adipose differentiation-related protein/perilipin; FASN, fatty acid synthase; IRS2, insulin receptor substrate 2; ChREBP, carbohydrate response element binding protein, PDK4; pyruvate dehydrogenase kinase 4; DGKD, diacylglycerol kinase δ. N4, 5mM 4 hours; H4, 18mM 4 hours, N24, 5mM 24 hours; H24, 18mM 24 hours.

Changes in gene expression between the control and treated cells were limited to a suppression of PDK4 at 4 hours and the induction of ChREBP and DGKD at 24 hours – no change in SREBP-1c abundance was observed. However there were also increases in the abundance of 5 transcripts between 4 and 24 hours in the high glucose treated cells, with ADFP, FASN, IRS2, CHREBP and PDK4 all being upregulated. There was also a 2.78-fold increase in PDP2 expression between 4 and 24 hours in the control samples. (Table 5.3.1).

5.4 Discussion

These results demonstrate that ChREBP may be able to induce lipogenic gene expression *in vitro,* independently of increases in SREBP-1c mRNA abundance, which could contribute to raised IMTG content through *de novo* lipogenesis. It was previously considered controversial to assume that there was any significant capacity for *de novo* lipogenesis in myocytes. However, the consensus has begun to shift in recent years due to *in vitro* studies showing that it could play a small, but important

role in fat balance (Aas *et al.*, 2004). A number of studies had previously established that glucose alone is capable of inducing the expression of HKII, FAS and ACC, via SREBP-1c, which leads to an increase in lipogenesis, and potentially IMTG concentrations (Guillet-Deniau *et al.*, 2004; Meugnier *et al.*, 2007).

In the present study, ChREBP mRNA expression was only observed in the high-glucose treated cells after 24 hours (Table 5.3.1), this made the calculation of a fold-change value impossible, although it did confirm an effect for high-glucose treatment, which indicates that perhaps in the absence of insulin, only prolonged incubation in a high-glucose medium, can initiate its transcription. LXRα is a likely transcriptional regulator of ChREBP in this situation, as has been described in liver (Cha & Repa, 2007). Clarification is still required as to whether LXRα binds glucose directly to activate ChREBP, but in the absence of insulin, which mediates lipogenic gene expression via SREBP-1c, the data in this study suggest a reliance upon ChREBP for the induction of lipogenic genes. SREBP-1c expression was found to be unchanged in this study.

This study also demonstrated the induction of a ChREBP target gene, fatty acid synthase (FAS) (Dentin *et al.*, 2004) between 4 and 24 hours in high-glucose treated cells (Table 5.3.1). Fatty-acid synthase is responsible for synthesizing long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH (Semenkovich, 1997). Oversupply of glucose, followed by an increased flux through the glycolytic pathway, and expansion of the TCA cycle, could increase the cytosolic concentrations of citrate. Citrate is a starting point for the synthesis of malonyl-CoA, which inhibits β-oxidation and is also the precursor of fatty-acid synthesis, via ACC and FAS. This sets in motion a process through which an increase in fatty-acyl-CoA, DG and TG concentrations, would lead to the accumulation of IMTG (Figure 5.4.1). This process would also explain the induction of adipose differentiation-related protein (ADFP) (Table 5.3.1), also known as perilipin, which is responsible for coating lipid droplets. Its increase in expression between 4 and 24 hours would help in sequestering intramyocellular lipids to reduce lipotoxicity (Phillips *et al.*, 2005b). ADFPs expression is under the control of PPARγ in adipose tissue (Dalen *et al.*, 2004), and similar

systems may regulate its expression in myocytes. It has also recently been demonstrated that LXR agonists directly induce ADFP expression in primary hepatocytes, suggesting another important link between hyperglycaemia and lipid storage (Kotokorpi *et al.*, 2010). ADFP is also down-regulated in the skeletal muscle of diabetic subjects which may be linked to the development of insulin-resistance through reduced lipid clearance (Phillips *et al.*, 2005a).

Diacylglycerol glycerol kinase δ (DGKD) was significantly upregulated at 24 hours in high-glucose treated cells, compared to the 24 hour control. DGKD has been implicated in the development of hyperglycaemia induced insulin resistance, through its substrate diacylglycerol (Chibalin *et al.*, 2008). Diacylglycerol is a precursor in the development of triglycerides, and prolonged increases in cellular DAG concentrations are associated with the accumulation of intramyocellular triglycerides, reductions in insulin PKC mediated signalling and insulin resistance (Timmers *et al.*, 2008) (Itani *et al.*, 2002). DAG is also phosphorylated to produce phosphatidic acid which acts as a secondary messenger, in a range of metabolic pathways. DGKD is responsible for this phosphorylation event. DGKs are able to deplete DAG at various intracellular sites, and may reduce the potential for the developing insulin resistance. One study found that 96 hours of high-glucose (25mM) incubation downregulated DGKD expression in myotubes cultured from healthy individuals (Chibalin *et al.*, 2008). Conversely this study observed that 24 hours high-glucose treatment lead to a 2.12 fold induction ($p<0.05$). It could be the case that since the study by Chibalin *et al* used higher glucose concentrations, and for a much longer period, that a more 'diabetic' phenotype is observed in the cell, with a failure of such protective mechanisms, as observed in the present study.

PDK4, which regulates the activity of the pyruvate dehydrogenase complex (PDC) is suppressed by insulin (Majer *et al.*, 1998) and upregulated by glucose deprivation and high-fat feeding (Abbot *et al.*, 2005) (Tsintzas *et al.*, 2007). A number of pathways coordinate these changes, with the involvement of the FOXO in the insulin response and PPARα, PGC-1α and ERR mediating the effects of fatty acids (Furuyama *et al.*, 2003) (Wende *et al.*, 2005). In this study PDK4 was transiently

suppressed (-1.58 fold) in the high-glucose treated myotubes at 4 hours, which suggests that despite a lack of insulin stimulation/PI3K signalling, there is a mechanism which attempts to utilise the available carbohydrate, by increasing PDC flux (Mandarino *et al.*, 1993). Conversely PDK4 expression was significantly upregulated at 24 hours which indicates that, a switch in fuel substrate had occurred or at least dependence on glucose was reduced. Mechanisms for this induction of PDK4 could be via FOXO, but since there was a lack of insulin signalling in all treatments, under that hypothesis its expression would be expected to rise in each group. Therefore PPARα induction of PDK4, as an indirect result of increased ligand-binding through lipid accumulation, is an alternative regulatory pathway. This would act as a brake on the accumulation of IMTG, and increased β-oxidation of fatty acids - possibly constituting a semi-futile cycle of IMTG synthesis followed by β-oxidation, preventing lipotoxicity. IRS2 is involved the control of peripheral insulin sensitivity (Withers *et al.*, 1998; Previs *et al.*, 2000), and it's upregulation in high-glucose treated myotubes, between 4 and 24 hours, are potentially also a compensatory pathway for the development of insulin-resistance in high-glucose treated cells - its expression being regulated by PPARγ (Smith *et al.*, 2001).

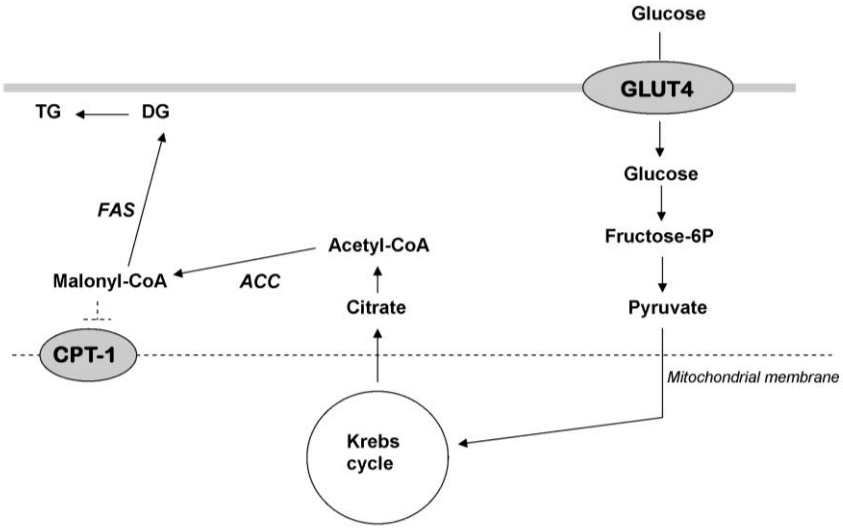


Figure 5.4.1 Hypothesised routes via which prolonged excess glucose availability may cause insulin resistance and lipotoxicity in skeletal muscle. Excess glucose or supply, leads to increased production of acetyl-CoA, which is then converted into free-fatty acids by ACC. Under these conditions, malonyl-CoA inhibition of CPT-1 would prevent β-oxidation, leading to an expansion of the FA-CoA, triacylglycerol and diacylglycerol pool. Malonyl-CoA could also serve as a substrate for fatty acid synthesis worsening this problem. Two potential mechanisms to prevent lipotoxicity during hyperglycaemia were revealed in this study; i) sequestering DG through coating lipid droplets using ADFP for storage, or ii) deplete DG through DGDK activity. B-oxidation may also be increased latterly, but the rate of this would be balanced against the concentrations of malonyl-CoA.

Future work should examine the global expression profiles, *in vivo*, to determine whether this hypothesis can be sustained when looking more generally at the networks involved, and also whether these situations can be maintained over longer-durations. Prospective studies, should examine IMTG synthesis directly, and also use a comparative approach with myotubes grown from diabetic subjects – with measurements of fat oxidation and glucose uptake to establish any changes in these pathways. It's possible that disruption to this protective system during prolonged hyperglycaemia could play a role in the development or maintenance of local insulin resistance in skeletal muscle, during T2DM.

In conclusion, the present study shows that 24 hours high-glucose treatment of primary myotubes, leads to changes in the expression of metabolic gene expression, associated with lipogenesis, in particular the transcription factor ChREBP. These changes may reflect a mechanism which promotes increasing accumulation of IMTG during hyperglycaemia *in-vivo*. However, high-glucose treatment also elicits responses in myotubes which may be capable of mitigating the effects of lipotoxicity, through depletion of diacylglycerol, lipid storage and increased β-oxidation. Understanding how these pathways might be altered in obese insulin-resistant muscle could explain the link between IMTG and insulin resistance.

Chapter 6

Skeletal muscle metabolic gene expression is not affected by dichloroacetate mediated modulation of substrate utilisation

6.0 Skeletal muscle metabolic gene expression is not affected by dichloroacetate mediated modulation of substrate utilisation

6.1 Introduction

The metabolic rate in human skeletal muscle is tightly regulated - with the control of flux through metabolic pathways being essential as cells adapt rapidly to fluctuations in energy intake and expenditure, and also to changes in substrate availability. These changes can be brought about through changes in enzyme activity, but often require stabilisation for longer periods. This can be accomplished through adaptive changes in the transcriptional activity of metabolic genes, due to interactions with transcription factors, which can elicit increased or decreased cellular responses to energetic demands.

It has been well established that insulin regulates the activity of PDC (Mandarino *et al.*, 1987) and recent studies have shown that this results from changes in the expression of pyruvate dehydrogenase kinase 4 (PDK4) mRNA, whereby it is upregulated during starvation (Pilegaard *et al.*, 2003; Spriet *et al.*, 2004) and suppressed with insulin infusion (Majer *et al.*, 1998; Lee *et al.*, 2004). Conversely, Hexokinase II (HKII) and its transcriptional regulator, Sterol-regulatory-element-binding protein-1c (SREBP-1c), mRNA and protein expression are reduced during fasting (Bizeau *et al.*, 2003; Gosmain *et al.*, 2005) .

Forty-eight hours starvation downregulates SREBP1c and HKII whilst PDK4 is upregulated – a response which was not dependent on the phosphorylation status of Akt and Forkhead box 01 (FOXO1), or without changes in the expression of transcription factors or genes involved in fat metabolism (Tsintzas *et al.*, 2006), which indicates that genes involved in carbohydrate oxidation respond rapidly and independently of those in fat oxidation. Further research indicates that elevating levels of circulating free fatty-acids, through infusion of intralipid and heparin, attenuates the insulin induced suppression of PDK4 (Tsintzas *et al.*, 2007). These previous studies caused rapid alterations to metabolic gene expression; however they have all modified insulin signalling along with fuel availability. It has not yet been

assessed whether these changes to metabolic gene expression can be induced by simply increasing PDC flux and switching fuel use – towards glucose oxidation – without altering plasma substrate availability and insulin signalling. An improved understanding of how closely fuel use is coupled to the expression of metabolic genes; would provide a better model for many metabolic diseases, such as diabetes and obesity.

DCA is an organohalide analogue of pyruvate which increases flux through the PDC via inhibition of PDK. Under normal conditions the PDC catalyzes the conversion of pyruvate to acetyl coenzyme A (CoA). PDC activity is modulated by reversible phosphorylation through PDK, which inactivates the PDC, and PDC phosphatase (PDP) which dephosphorylates PDC restoring catalytic activity (Stacpoole *et al.*, 1998). PDK is activated by NADH and Acetyl CoA and inhibited by pyruvate and DCA. As an analogue of pyruvate, DCA binds to PDK and prevents it from phosphorylating PDC into its inactive form, this leaves the PDC in its switched 'on', catalytic, un-phosporylated active state. Administration of DCA in skeletal muscle at rest exhibits certain key features including; pyruvate dehydrogenase complex activation, increased carbohydrate oxidation, and elevation of acetylcarnitine.(Timmons *et al.*, 1998; Constantin-Teodosiu *et al.*, 1999; Knoechel *et al.*, 2006). Constantin-Teodosiu et al have extensively researched the effect of DCA in human muscle during exercise (Constantin-Teodosiu *et al.*, 1999) and concluded that the observed delay in accumulation of fatigue related metabolites, during exercise with DCA infusion, is due to increased availability of acetyl groups to the TCA cycle –in the form of acetylcarnitine -, rather than through expansion of the Tri Carboxylic-Acid Cycle Intermediate (TCAI) pool itself. DCA was selected for use in this study because of its capacity to increase PDC flux and glucose oxidation without changing substrate availability and glycolytic flux.

The aim of this study was to assess whether alterations in metabolic gene expression, in human skeletal muscle, could be produced by changes in fuel use and *not* plasma substrate availability. Through solely altering fuel selection, and increasing PDC flux, it was hypothesized that there would be no changes in metabolic gene

expression resulting from a short term (90 minutes - to limit the effects of fasting) DCA infusion.

6.2 Materials and methods

Subjects

Ten healthy non-obese men were recruited. Mean age was 29.2 ± 1.56 years with a body mass index (BMI) of 24.7 ± 0.85kgm^{-2}. They attended the laboratory on two occasions after a 12 hour overnight fast, having abstained from strenuous exercise and alcohol intake for the previous 24 hours. Subjects with any condition, disease or drug that would affect metabolic rates were excluded from these studies. All procedures carried out in this study were performed according to the Declaration of Helsinki and approved by the University of Nottingham Medical School Ethics Committee, with informed written consent obtained from all subjects.

Study protocol

Subjects attended the laboratory on 2 occasions for a 90 minute infusion, in random order, after a 12 hour overnight fast. After a 60 minute basal period an infusion of sodium dichloroacetate (DCA) was given at a rate of 50 mg kg^{-1} min^{-1} over 90 minutes. The infusate was prepared by the sterile supplies unit at the University Hospital Nottingham Pharmacy. On a separate occasion, subjects received a 0.9% saline infusion which served a control (CON). Each study visit was at least two weeks apart. Studies took place in a temperature-controlled room (23°C) with subjects wearing a T-shirt and shorts only. On arrival, subjects rested supine for 30 minutes whilst two intravenous cannulae were inserted, the first into the antecubital vein for the infusion, the second retrogradely into a vein on the dorsal aspect of the hand for blood sampling. A slow running infusion of 0.9% saline was used to keep this patent. The hand was placed in a warm air box (50-55°C) to obtain arterialised blood samples (McGuire *et al.*, 1976).

Muscle biopsies

Muscle biopsies were taken immediately before the start of the infusions, 30 minutes into the infusion and 3 hours from the baseline (90 minutes post-infusion – to allow for changes in transcription factor localisation to occur). Muscle samples were obtained from the vastus lateralis under local anaesthetic using percutaneous Bergstrom needles (Bergstrom, 1975). The samples were immediately frozen and stored in liquid nitrogen for subsequent analysis of gene expression.

Indirect calorimetry

Respiratory exchange was measured during the last 30 minutes of the basal period, the first 30 minutes of the infusion and the last 30 minutes of the study period. Continuous recordings of oxygen consumption and carbon dioxide production were made using a computerised flow-through canopy gas analyser system using either a SensorMedics 2900 metabolic cart (SensorMedics, CA) or Europa GEM (gas exchange monitor, Europa Scientific Ltd, UK) (Frayn, 1983; Nicholson *et al.*, 1996). The same analyser was used for each subject. Calculation of resting energy expenditure (REE) and estimation of carbohydrate and lipid oxidation rates were made using indirect calorimetry (Frayn, 1983).

Analytical procedures

Blood samples were drawn during the baseline period and every 30 minutes during the study period (up to 3 hours) and used for the measurement of intermediary metabolites and hormones. Plasma and serum were separated using low-speed centrifugation (15 min at 3000 *g*). Plasma free fatty-acid (FFA) concentrations were measured by an enzymatic method (NEFA C, WAKO-Chemicals GmbH, Germany,). Serum insulin concentrations were measured using a radioimmunoassay (Coat-a Count; Diagnostic Products Corporation) and whole blood glucose and lactate concentrations were analysed using a Yellow Springs analyser (YSI 2300 Stat plus; Yellow Springs Industries, OH, USA).

RNA preparation and quantification

Total RNA was isolated from muscle biopsies using Trizol (Invitrogen), and further purified using RNeasy RNA clean up columns (Qiagen). RNA concentration was determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Delaware, USA).

RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). The mRNA levels were quantified using Real-time quantitative reverse transcriptase – polymerase chain reaction (RT-qPCR), performed in an ABI Prism 7000 sequence detection system. Primer and FAM/TAMRA labelled probes were designed using Primer Express® Software v2.0 (Applied Biosystems); sequences are shown in Supplemental Table 2.2.1. The standard curve method was used; with acceptable units for the slope, between -3.2 and -3.6 and R^2 values of more than 90%. The probes and primers for the non-variant reference gene, Hydroxymethylbilane synthase (HMBS), were purchased from Applied Biosystems. All values were normalised by dividing the target gene by HMBS.

Protein analysis by immunoblotting

The protein lysates were extracted from the organic phase using Trizol (Invitrogen) and then the pellets were redissolved in 7M urea, 100mM DTT, 50mM Tris and 4%SDS. Protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). Protein (50µg) was subjected to SDS-PAGE in a Bio-Rad (Hercules, CA) miniature slab gel apparatus (Mini-Protean). Electrotransfer of the proteins from gel to PVDF was performed for 120 minutes at 200 mA. Non-specific protein binding was reduced by blocking the PVDF in blocking buffer (5 % non-fat dry milk, 10 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl, 0.02% Tween 20). The PVDF membrane was subsequently incubated with anti-phosphoAkt (Ser^{473}) Mouse mAb (#4051, Cell Signalling Technology) antibody, in a 1:1000 dilution of 1% blocking buffer overnight at 4°C. The blot was then incubated with an HRP conjugated secondary antibody (P0447, DAKO) in a 1:2000 dilution of 1% blocking buffer for 2 hours at room temperature. This procedure was repeated for total Akt (Ab #9272, Cell signalling) and the secondary Ab (P0217, DAKO). α-actin was used to normalise protein expression

levels using an α-actin primary (A2066, Sigma-Aldrich). The blots were developed using ECL reagents (GE Healthcare, Bucks, UK), and exposed to Amersham Hyperfilm ECL (GE Healthcare). Band intensities were quantified by optical densitometry, using Bio-rad, Quantity One software (Hercules, CA).

Statistical analysis

Values are given as means ± SEM. Comparisons in; metabolite concentrations, substrate oxidation rates, gene expression and protein expression - both between and within treatments - were made by 2-way repeated measures ANOVA (applying a Bonferroni post-*hoc* test), using SPSS version 15.0 (SPSS Inc, Chicago IL). The threshold for significant change was $P<0.05$.

6.3 Results

Plasma hormones and metabolites

Blood glucose levels were significantly reduced by 0.26 ± 0.07 mmol L^{-1} ($P<0.05$) in DCA and by 0.14 ± 0.06 mmol L^{-1} ($P<0.05$) in the control infusion. At 3hrs after the start of the infusion, blood glucose in DCA was lower than CON by 0.16 ± 0.05 mmol L^{-1} ($P<0.05$, Table 6.3.1). Blood lactate concentrations fell in both infusions; by 0.24 ± 0.05 mmol L^{-1} ($P<0.05$) in DCA, and 0.12 ±0.03 mmol L^{-1} in CON ($P<0.05$), with DCA decreasing significantly more than CON ($P<0.001$, Table 6.3.1). Serum insulin concentrations dropped significantly during the DCA infusion by 1.02 ± 0.29 mmol L^{-1} ($P<0.05$). The CON infusion induced a trend for decreasing concentration but it was not significant ($P=0.08$), neither were the serum insulin concentrations significantly different at 3 hours post infusion (Table 6.3.1). FFA concentrations increased during the saline infusion by 0.07 ± 0.03 mmol L^{-1} ($P<0.05$). DCA was associated with a significant fall in FFA concentrations at 90 minutes, by 0.12 ±0.05 mmol L^{-1} ($P<0.05$), followed by an increase at 3 hours, giving no significant overall change ($P=0.76$) (Table 6.3.1.)

Table 6.3.1 Blood and plasma metabolite concentrations at baseline and during the intervention.

	Baseline	1 hour	3 hours
Blood glucose (mmol/L)			
DCA	4.56 ± 0.11	4.39 ± 0.10*	4.30± 0.07*†
CON	4.60 ± 0.07	4.43 ± 0.06*	4.46 ± 0.04*
Blood lactate (mmol/L)			
DCA	0.46 ± 0.05	0.31 ± 0.01*	0.22 ± 0.01*†
CON	0.55 ± 0.04	0.52 ± 0.03	0.44 ± 0.03*
Serum insulin (pmol/L)			
DCA	24.0 ± 3.0	19.8 ± 4.2	18.0 ± 2.4*
CON	24.6 ± 3.0	21.0 ± 2.4	19.8 ± 2.4
Plasma Free fatty-acids (mmol/L)			
DCA	0.76 ± 0.05	0.66 ± 0.04*	0.75 ± 0.04
CON	0.70 ± 0.04	0.73 ± 0.03	0.77 ± 0.04*

Data are means ± S.E., n=10, *P<0.05 vs Baseline ,† P<0.05 vs CON

Whole body substrate metabolism

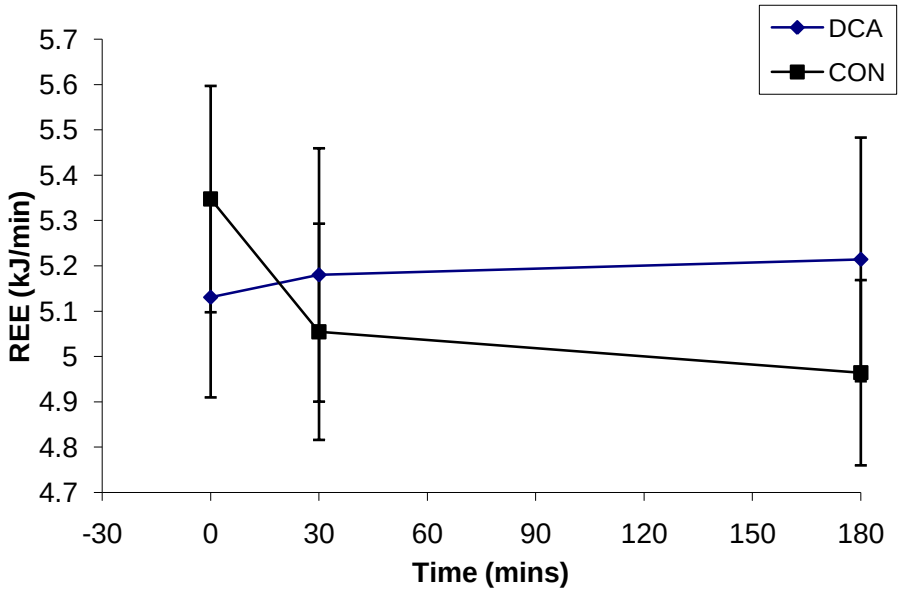
Carbohydrate oxidation was increased by DCA (P<0.05) at 3 hours with no change in the CON infusion (Table 6.3.2). Lipid oxidation showed no change in either DCA or CON (Table 6.3.2). REE was significantly reduced in CON (0.38± 0.09 kJmin^{-1}; P<0.05), whereas the DCA infusion did not change the REE. The two groups were not significantly different at any time. (Figure 6.3.1)

Table 6.3.2 Whole body substrate metabolism. CHO Oxn; Carbohydrate oxidation rate, FAT Oxn; Fat oxidation rate.

	Baseline	30 minutes	3 hours
CHO Oxn (g min-1)			
DCA	0.16 ± 0.02	0.13 ± 0.02	0.19 ± 0.02*
CON	0.16 ± 0.02	0.16 ± 0.02	0.15 ± 0.02
FAT Oxn (g min-1)			
DCA	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
CON	0.05 ± 0.01	0.044 ± 0.01	0.05 ± 0.01

Means ± S.E., n =10, *P<0.05 vs Baseline. No significant differences DCA vs CON

Figure 6.3.1 Resting Energy Expenditure (REE) at baseline and 30, 180 minutes post-infusion.



Expression of metabolic genes in skeletal muscle

PDK4 was unchanged in both CON and DCA, with no significant interaction between time and treatment. Uncoupling protein-3 (UCP3) showed no treatment effect, but it was significantly upregulated at 3 hours in the CON trial, and remained unchanged in DCA. A similar pattern was observed in peroxisome proliferator-activated receptor γ co-activator-1α (PGC1α) with no difference between the treatments. All other transcripts were unchanged in both interventions. Baseline values for all genes were not significantly different (Table 6.3.3).

Table 6.3.3 mRNA expression (fold change from baseline) for CON and DCA infusions in skeletal muscle.

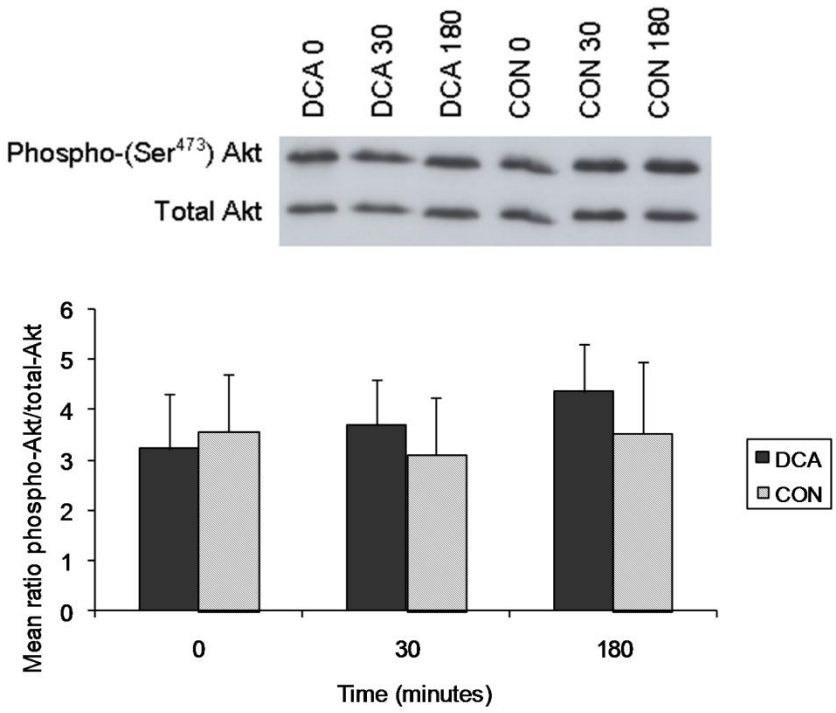
Gene expression: fold change		Time (minutes)	
		30	180
PDK4			
	DCA	0.94 ± 0.23	1.24 ± 0.34
	CON	1.52 ± 0.22	2.66 ± 0.75
PDK2			
	DCA	1.11 ± 0.08	0.98 ± 0.08
	CON	0.98 ± 0.21	1.23 ± 0.42
HKII			
	DCA	1.02 ± 0.16	1.18 ± 0.19
	CON	1.11 ± 0.19	0.87 ± 0.13
CPT1			
	DCA	1.25 ± 0.21	1.00 ± 0.10
	CON	1.28 ± 0.25	1.37 ± 0.24
UCP3			
	DCA	1.24 ± 0.18	1.20 ± 0.15
	CON	1.42 ± 0.25	1.69 ± 0.22*
SREBP1c			
	DCA	1.01 ± 0.10	1.12 ± 0.21
	CON	1.14 ± 0.18	1.11 ± 0.22
PPARα			
	DCA	0.94 ± 0.12	1.01 ± 0.13
	CON	1.12 ± 0.14	1.16 ± 0.17
PPARδ			
	DCA	0.93 ± 0.06	0.94 ± 0.15
	CON	1.33 ± 0.17	1.393 ± 0.20
PGC1α			
	DCA	1.23 ± 0.21	1.08 ± 0.14
	CON	1.33 ± 0.12	1.84 ± 0.18*

All values are reported as means ± S.E. n=10, *P<0.05 vs Baseline. No significant differences DCA vs CON

Protein expression

There were no significant differences in total skeletal muscle protein Akt expression or Ser^{473} phosphorylation, either between treatments or during the infusions (Figure 6.3.2).

Figure 6.3.2 Akt phosphorylation. Data are means ± S.E. ratio of phosphorylated-Akt (Ser^{473}) divided by total Akt for DCA and CON at baseline (0 minutes) and 30 and 180 minutes after the start of the infusion. One representative immunoblot showing phosphorylated-Akt and total-Akt.



6.4 Discussion

Studies have shown that the regulation of glucose and fatty-acid metabolism is a multi faceted and highly integrated process (Randle, 1998), requiring both changes in enzyme kinetics and gene expression. Skeletal muscle is the major site for insulin stimulated glucose disposal, and the pathways which regulate carbohydrate metabolism have been shown to respond to changes in substrate availability and hormonal signalling with secondary adaptations in gene expression (Arkinstall *et al.*, 2004). There is no evidence that acute activation of the PDC can be accomplished without changing substrate availability or metabolic gene expression.

The DCA infusion in this study showed an increase in carbohydrate utilisation and changes in blood metabolites which were consistent with previous studies

involving DCA (Timmons *et al.*, 1998; Constantin-Teodosiu *et al.*, 1999). There were sustained and significant decreases in blood lactate, probably due to increased conversion of pyruvate to acetyl-coA via the PDC (Stacpoole *et al.*, 1998), accompanied by an increase in the whole body carbohydrate oxidation rate. In CON, the changes in blood glucose, serum insulin and FFA's are small, but in accordance with continued fasting. The confirmation by immunoblotting (Figure 6.3.2) that there were no changes in insulin signalling, provides evidence that changes in insulin stimulated glucose uptake via GLUT4, were not a factor in either infusion. The short duration of this study was intentional, to limit the effects of fasting on gene expression.

Human intervention studies adopting a wide range of approaches including insulin & adrenaline infusions (Tsintzas *et al.*, 2007), changes in dietary composition (Chokkalingam *et al.*, 2007) and starvation (Tsintzas *et al.*, 2006), have produced a wealth of data showing alterations in metabolic gene expression. Recent studies have shown that HKII is sensitive to alterations in substrate availability (Tsintzas *et al.*, 2007), and yet it was unchanged in this chapter, which suggests that changes in the expression of glycolytic genes are not required for short term increases in carbohydrate oxidation. The transcriptional activator of HKII, SREBP-1c was also unaffected. The expression of PDK2, the site where DCA is kinetically most effective, was also unchanged during both infusions, however, given that its basal level of expression and half-life are longer than that of PDK4, it might be sensible to expect a smaller role in the acute regulation of PDC flux (Huang *et al.*, 2002). PDK4 is a gene which plays a significant role in cellular adaptation to alterations in carbohydrate availability and oxidation, and other studies have reported this (Huang *et al.*, 2002), mainly through its close and inverse relationship with insulin (Lee *et al.*, 2004). However, as anticipated, there were no large decreases in serum insulin concentrations during this study, or any changes in PDK4 expression. Despite the potential action of DCA on PPARα - via changes in expression (Walgren *et al.*, 2005) and *trans*-Activation (Maloney & Waxman, 1999) - there was no change in the mRNA content of the PPARα and δ in DCA or CON. However, the PPAR co-activator, PGC1α, did increase significantly in CON, but not in DCA treated subjects. PGC1α

mRNA expression has previously been shown to increase during starvation (de Lange *et al.*, 2006) , and with weight loss (Gastaldi *et al.*, 2007). DCA may have had the effect of blocking any fasting induced increase in PGC1α mRNA, through increasing carbohydrate utilisation, preventing the cell from sensing a physiological negative energy-balance normally associated with saline infusions following an overnight fast. Wende et al have shown that PGC1α stimulates the expression of PDK4 (Wende *et al.*, 2005) and Zhang et al (Zhang *et al.*, 2006) have proposed a mechanism involving the Estrogen-related receptor (ERR) for induction of PDK4. It is likely that there is a complex interplay between a number of transcription factors in the regulation of PDK4 including PPARα, PGC1a, ERRα and Liver X receptor (LXR) (Sugden & Holness, 2003; Wende *et al.*, 2005).

Among the other potential targets for PPARs, CPT1 was unchanged in this study whilst UCP3 had contrasting results between the two treatments. An upregulation in UCP3 mRNA expression was seen in CON, which would be expected as a response to continued starvation. In contrast, in the DCA trial UCP3 expression was unaltered. One potential role of UCP3 that has been suggested is the export of fatty-acid anions out of the mitochondria (Nagy *et al.*, 2004). With decreased reliance on fat oxidation in DCA compared to CON it may be the case that UCP3's role is diminished (de Lange *et al.*, 2006). DCA did not cause any changes in gene expression per se, but subtlety reduced the effects of fasting. This provides evidence for the concept that PDC flux can be independently altered in the short-term, without changing the expression of metabolic genes.

Chapter 7

General Discussion and Conclusions

Chapter 7.0 General discussion and conclusions

The human body is remarkable in its ability to adapt to changes in energy balance and substrate availability. It does this through multi-level regulatory processes; from the smallest scale of the genome, through the activity of enzymes, and the coordination of organ systems, right up to the behaviour of the individual. Part of this response involves control of the transcriptional activity of metabolic genes. Metabolic gene expression is altered in response to changes in the external and internal environment (Mutch *et al.*, 2005), with the aim of maintaining the balance between energy intake and energy expenditure. Despite this simple goal, the systems which govern energy balance are highly complex, which confers both great resilience and flexibility. However, this same complexity makes understanding the process through which homeostasis is maintained, or how disease states arise, very difficult. Fortunately, the knowledge base surrounding metabolic networks has begun to grow, in parallel with improvements in technology which have made it possible to characterize and quantify the expression of metabolic genes.

Metabolic diseases have become more prevalent with the increasing energy intake and decreased energy expenditure associated with industrialisation, to the extent that it is now considered an international health crisis, the resolution of which is a priority (Popkin & Doak, 1998). This thesis has focussed on the problem of understanding how metabolic diseases arise, and also how metabolic gene expression changes in response to variations in nutrient supply. The studies in chapters 3 and 4 examined the role of postprandial fat oxidation in the adipose tissue of obese subjects, whilst chapters 5 and 6 are concerned with substrate utilisation in skeletal muscle.

Chapter 3.0 in this thesis demonstrated that although changes in gene expression are different between dietary and fat-oxidising groups, during calorie restriction (CR), there is no fundamental difference in weight loss. This not only implies that caloric restriction is a greater driver of weight loss than dietary composition and postprandial fat oxidising capacity, but also that gene expression here is playing a secondary role to the environment. The observed adaptations in

gene expression such as downregulation of HSL and ATGL, are ways of regulating or possibly adapting to changes in fuel use in response to weight loss, but are not in themselves driving weight loss. Further studies in this area, would benefit from the introduction of a post-diet postprandial fat oxidation test meal, to examine whether low –fat oxidisers on a high fat diet, and high-fat oxidisers on a low fat diet actually change their postprandial fat oxidising capacity after this period, since these two groups showed the largest changes in metabolic gene expression, despite having the same weight loss profiles.

Chapter 4 provided a continuation of the theme of postprandial fat oxidation with the assessment of global gene expression profiles. The initial PCA analysis confirmed that there were differences between the high- and low-postprandial fat oxidising populations, and further statistical analysis and filtering provided a set of genes which offered a central role for GLUT4, fatty-acid synthase (FASN) and aquaporin (AQP7) in the maintenance of the phenotype in adipose tissue. The main transcriptional factors mediating this were RXRα and SREBF1 (most likely SREBP-1c) which may have been regulated by LXR. The results in chapters 3 & 4 offer confirmatory findings such as the suppression of SREBP-1c expression during calorie restricted diets, but also offer new insight in the arena of postprandial fat oxidation, showing for the first time that gene expression profiles adapt differently to calorie restricted diets or varying fat content and that there are important transcriptomic differences between the adipose tissue of individuals with altered postprandial fat-oxidising capacity. This opens the door for future research to build upon by establishing, ideally via longitudinal studies, how such differences arise – however, caution should be used when trying to attribute causality in these situations since although it may meet with success at the very smallest scale; at this level any biological meaning is lost.

In the high-glucose study (chapter 5), ChREBP, which is a known activator of lipogenic gene expression, was upregulated along with its target FASN in high-glucose treated myotubes. This demonstrated the ability of chronic glucose oversupply to alter gene expression, in contrast to the results of acute dichloroacetate treatment

(chapter 6), where no changes were observed in the absence of measurable changes in glucose availability, but there was a clear switch from fat to carbohydrate oxidation. Taken together these results are a clear example of the multi-level regulation of metabolism which has been a recurring theme throughout this thesis. The high-glucose study also exposes potential mechanisms which could be responsible for the protection of myocytes from excess lipid accumulation during prolonged hyperglycaemia. These pathways all involved methods of lipid clearance through; perilipin induced sequestration into lipid droplets, depletion into phosphatidic acid by diacylglycerol kinase, and increased β-oxidation.

The diverging regulation of lipogenic gene expression was demonstrated in both the fat-oxidation studies and the high-glucose treatments. With the transcriptional regulators SREBP-1c and ChREBP both appearing to induce the expression of FASN- this is a clear example of how the expression of a gene can be induced in quite different circumstances, depending on substrate supply, disease state and tissue type. Furthermore the contribution of RXRα, displays the potential for synergistic interactions, which may also act as 'fail-safes' for important biological mechanisms should one pathway be compromised. The high level of redundancy in biological systems, and the pleiotropic effects of many metabolic genes forms the basis of these activities (Tononi *et al.*, 1999), which require researchers to must step back and suspend dualistic thinking when examining these problems (Van de Vijver, 2009).

Early linkage studies proved to be very successful in describing causal relationships between the genome and monogenic disease states, however despite the promise of association studies, the understanding of how genes might be involved in the pathogenesis of complex disease states remains limited. This could be the result of there being a vast number of rare polymorphisms, which are largely responsible for the development of a phenotype, or a small number of common polymorphisms, each of which make a small contribution towards a disease state or phenotype. In reality it is likely a mixture of these two situations, and hence a greater number of genomes will need to be sequenced across many different haplogroups before this question can be appropriately answered. Furthermore, genomes do not

exist in splendid isolation, and as Denis Noble has argued, we should not award any biological level (i.e. the genome) a privileged status (Noble, 2008). The work in this thesis has attempted to use this approach, by looking at how feed forward and feedback loops are integrated to maintain a dynamic equilibrium in the internal environment. eQTL analysis (chapter 4) integrated transcriptomic and genomic datasets and was successful in associating SNPs with gene expression, although interpretation of the data was complicated by the difficulty of resolving *'biased'* lists of differentially expressed genes from the potential false positives, and there is insufficient knowledge as to the operation of the genome at this stage to form strong hypotheses for these associations or to discard them entirely. Instead genes which were differentially expressed were linked with metabolite concentrations, such as the correlation between RXRα expression and fasting blood glucose concentrations. This type of data place greater emphasis on the interaction between nutrients and genes through the action of transcription factors.

The scientific community is increasingly aware of the contribution that methylation and gene imprinting has upon the phenotype; intergenerational environmental effects can now be observed that would have been dismissed as Lamarkian nonsense only 15 years ago. It is important not only to recognise that metabolic processes are regulated at multiple levels but that we should avoid being too hasty in trying to assign causality to very complex situations. One recent paper 'Fatness leads to inactivity, but inactivity does not lead to fatness: a longitudinal study in children (EarlyBird 45) (Metcalf *et al.*, 2010) suggested that inactivity is the cause of increased adiposity and not vice-versa. However, whilst the findings of this investigation are robust, there is plenty of evidence that obesity can be caused by both decreased energy expenditure or increased calorific intake, and most commonly a combination of both, which has been observed in developed countries. The concept of the gene itself is becoming rather outmoded, in light of evidence showing that alternative splicing and miRNA regulate transcriptional activity to such an extent that the idea of a gene has become rather diffuse. Exomic sequencing would provide a clearer picture of what is actually being transcribed from the genome, and as shown in

chapter 4 alternative splicing in SREBF1 may partly account for differences in fat oxidising capacity. The relevance in this case being that SREBP-1c activates lipogenic genes with greater efficiency than -1a, potentially improving lipid storage capacity.

An additional consideration for future work in modelling metabolic networks; is the importance of making sure that functional characterisation of transcripts is thoroughly carried out. When undertaking analysis of gene-expression data, it is necessary to use pathway analysis software to extract meaning from lists of transcripts, however it is vital that this should be used in conjunction with co-expression and GSEA analysis as was carried out in chapter 4, to prevent researchers from overlooking the importance of genes which are yet to be characterised, but could play important roles in metabolic disease and other physiological systems.

The development of obesity and diabetes will not have a clear step-by-step pathogenesis, since metabolic disease is distinct from monogenic diseases such as cystic fibrosis. Integration of multiple data sources will be required to model metabolic disorders properly, and also when applying this to diagnosis when the era of personalised medicine become practical (Mo & Palsson, 2009).

The scientific community should be prepared to see various mechanisms such as ectopic fat accumulation and altered adipokine release both being part of these metabolic disease states, and not necessarily preceding one another but rather emerging simultaneously in response to changes in energy intake and expenditure. This offers the possibility of multiple targets where treatments can be developed to counteract metabolic disease, but likely also requires pharmaceutical agents which are able to work less specifically in order to have any useful effect. Side-effects from anti-obesity or anti- diabetic, drugs will be hard to limit given the interdependence of nuclear receptor targets, and regulators should give serious consideration as to whether small but significant risks from a specific drug, really out-weigh the risks of prolonged metabolic disease.

The heterogeneity of disease states makes personalised medicine an attractive option, and the use of genotyping during treatment of patients is likely to become more common.

In summary this thesis shows that the human body is capable of adjusting metabolic gene expression quite considerably to counter or facilitate changes in substrate availability, but that it is also resilient in the short term to acute changes in flux through metabolic pathways. It also describes potentially important and novel mechanisms; the disruption of which could be linked to the progression of metabolic disease.

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Appendix I

Figure A: PPAR:RXR pathways. Unfiltered gene expression data (Chapter 4)

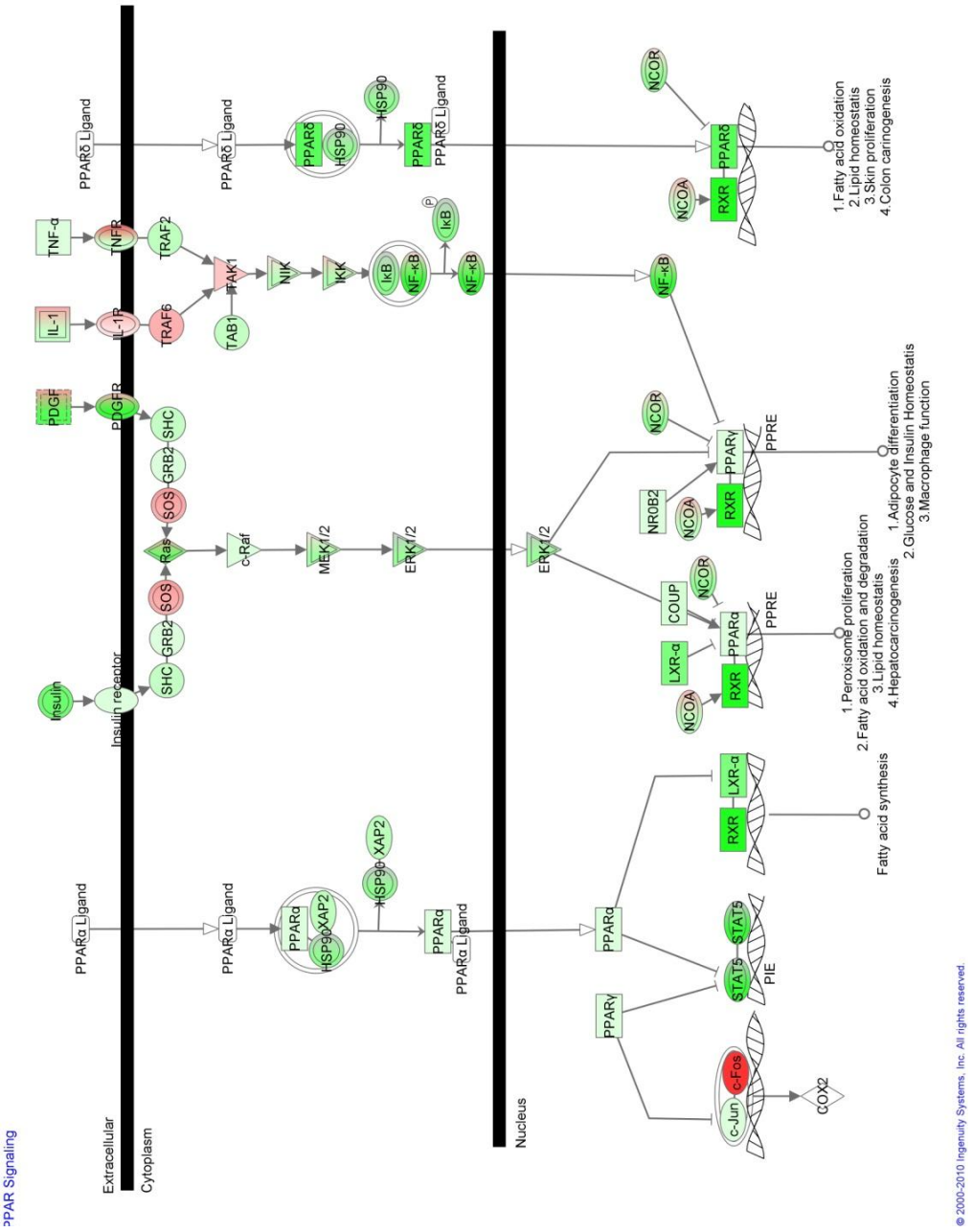

Figure B: LXR:RXR pathways. Unfiltered gene expression data (Chapter 4)

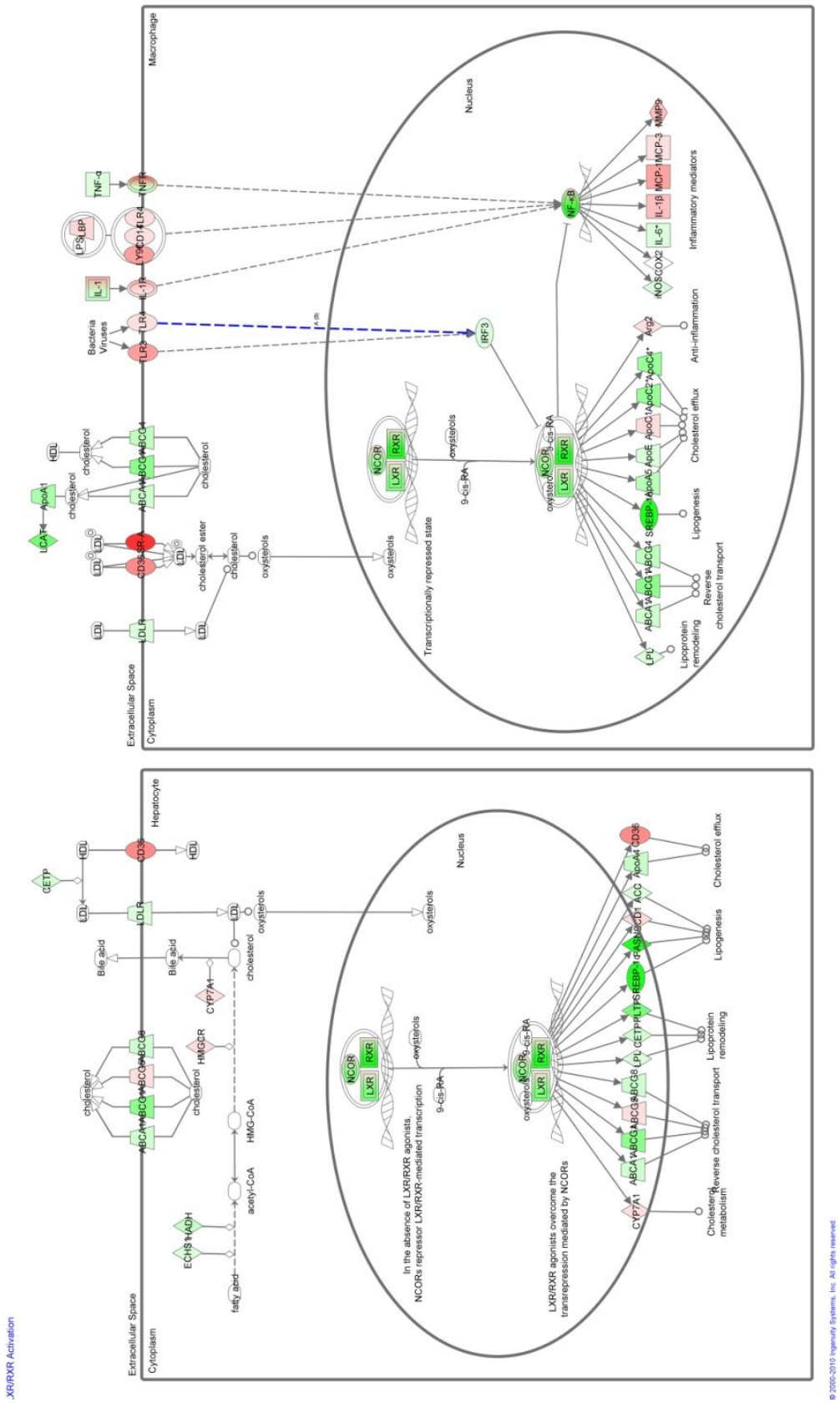

Table A. Differentially expressed genes. Fold-change>1.28 P<0.05 (Chapter 4)

Genesymbol	Fold change([HIGH] vs [LOW])	Regulation([HIGH] vs [LOW])	Genedescription	Genbank
ABCA6	1.3571911	up	ATP-binding cassette, sub-family A (ABC1),	AY028898
ADCY6	1.3187706	down	adenylate cyclase 6	BC064923
ADRA2A	1.3758698	down	adrenergic, alpha-2A-, receptor	AF284095
ADRB1	1.3116388	down	adrenergic, beta-1-, receptor	J03019
AGPAT1	1.2800417	down	1-acylglycerol-3-phosphate O-acyltransfer	BC090849
AKR1C3	1.5359242	up	aldo-keto reductase family 1, member C3 (	BC001479
ALDOC	1.3050004	down	aldolase C, fructose-bisphosphate	BC003613
AMAC1L2|AMAC	1.3596889	down	acyl-malonyl condensing enzyme 1-like 2	BC131696
ANGPT1	1.3551071	up	angiopoietin 1	BC152411
AOC3	1.2886319	down	amine oxidase, copper containing 3 (vascu	U39447
AQP7|AQP7P1|A	1.3710415	down	aquaporin 7 | aquaporin 7 pseudogene 1	BC119672|B
ARHGEF15	1.2846545	down	Rho guanine nucleotide exchange factor (I	BC036749
ATL3	1.297632	up	atlastin 3	AK090822
ATM|NPAT	1.3034247	up	ataxia telangiectasia mutated | nuclear p	U33841|X91
ATP5L|ATP5L2	1.3342676	up	ATP synthase, H+ transporting, mitochondr	BC015128|B
ATP5SL	1.3494071	down	ATP5S-like	BC013323|A
AVPI1	1.3722355	down	arginine vasopressin-induced 1	AF241786
BOK	1.3894626	down	BCL2-related ovarian killer	BC006203
BTBD2	1.4143441	down	BTB (POZ) domain containing 2	AF355797
BZW1	1.336499	up	basic leucine zipper and W2 domains 1	AL833518
C10orf10	1.4220606	down	chromosome 10 open reading frame 10	AB022718
C12orf35	1.4203917	up	chromosome 12 open reading frame 35	AB046771
C17orf65	1.3175187	down	chromosome 17 open reading frame 65	BC009448
C18orf32	1.4127433	up	chromosome 18 open reading frame 32	BC093004|A
C2orf40	1.3668827	down	chromosome 2 open reading frame 40	AY358601
C3orf63	1.3651072	up	chromosome 3 open reading frame 63	AF180425
C4orf18	1.295632	up	chromosome 4 open reading frame 18	BC043193|A
C6orf62	1.3063585	up	chromosome 6 open reading frame 62	BC040260
C6orf72	1.2847519	up	chromosome 6 open reading frame 72	AY358952
CACNA2D1	1.6213539	up	calcium channel, voltage-dependent, alph	BC117468
CALD1	1.3185304	up	caldesmon 1	M83216
CAPZA1	1.3126619	up	capping protein (actin filament) muscle Z-	BX648738
CASP4|LOC6437	1.3266383	up	caspase 4, apoptosis-related cysteine pep	AK057094|B
CCDC80	1.2886823	up	coiled-coil domain containing 80	AB266387
CD81	1.3852338	down		AB209380
CDC27	1.3093524	up	cell division cycle 27 homolog (S. cerevisia	BC011656
CHRM1	1.3090012	down	cholinergic receptor, muscarinic 1	BC022984
CISH	1.2879168	down	cytokine inducible SH2-containing protein	AF035947
CKB	1.2964231	down	creatine kinase, brain	AK290101
CLSTN2	1.3122133	down	calsyntenin 2	AY753303
CNTFR	1.30802	down	ciliary neurotrophic factor receptor	M73238
COPE	1.2884257	down	coatomer protein complex, subunit epsilo	BC007250
COX7B	1.2965676	up	cytochrome c oxidase subunit VIIb	Z14244
CP	1.3387864	up	ceruloplasmin (ferroxidase)	BC146801
CRIP2	1.2949564	down	cysteine-rich protein 2	AK091845
CSNK1A1	1.3429672	up	casein kinase 1, alpha 1	AF447582
CTDSP1	1.4229194	down	CTD (carboxy-terminal domain, RNA polym	AF229162
CTNNBIP1	1.3693366	down	catenin, beta interacting protein 1	AB021262
CYGB	1.3375119	down	cytoglobin	BC029798
CYP2F1	1.3748977	down	cytochrome P450, family 2, subfamily F, pol	J02906
CYP7B1	1.3155025	up	cytochrome P450, family 7, subfamily B, po	AF127090
DCUN1D1	1.423355	up	DCN1, defective in cullin neddylation 1, dc	AF292100
DSEL	1.2945799	up	dermatan sulfate epimerase-like	AF480435
DTX1|LOC10012	1.329856	down	deltex homolog 1 (Drosophila) | hypotheti	BC048216|A
EEA1	1.3565503	up	early endosome antigen 1	L40157
EPB41	1.4220278	up	erythrocyte membrane protein band 4.1 (e	AF156225
ERAP1	1.28302	up	endoplasmic reticulum aminopeptidase 1	AF222340
ERV3|ZNF117	1.6929694	up	endogenous retroviral sequence 3 (includ	M55422
ESAM|LOC10012	1.2865764	down	endothelial cell adhesion molecule | hyp	AY189281|A
F13A1	1.4683992	up	coagulation factor XIII, A1 polypeptide	BC027963
FAM126A	1.3657682	up	family with sequence similarity 126, mem	AL833296
FAM38A	1.2944229	down	family with sequence similarity 38, memb	D87071|BC0
FAM82B|NTAN1	1.2879077	up	family with sequence similarity 82, memb	AF151848
FASN	1.567724	down	fatty acid synthase	U29344
FBXO34	1.3953445	down	F-box protein 34	AF531436

Genesymbol	Fold change([HIGH] vs [LOW])	Regulation([HIGH] vs [LOW])	Genedescription	Genbank
FSCN1	1.2861162	down	fascin homolog 1, actin-bundling protein (	U03057
FXYD1|FXYD7	1.4315841	down	FXYD domain containing ion transport regi	AK289811
GDPD5	1.332104	down	glycerophosphodiester phosphodiesteras	BC018771
GMFB	1.3870455	up	glia maturation factor, beta	BX647679
GOLGA4	1.2939342	up	golgi autoantigen, golgin subfamily a, 4	U41740
GPR124	1.2888402	down	G protein-coupled receptor 124	AF378755
GPR146|LOC100	1.3736134	down	G protein-coupled receptor 146 | hypothet	BC014241|A
GPSN2	1.2967759	down	glycoprotein, synaptic 2	BC005384
GRINA	1.3535607	down	glutamate receptor, ionotropic, N-methyl [	AK127640
GRN	1.3576738	down	granulin	AF055008
GSPT1	1.2975861	up	G1 to S phase transition 1	BC009503
HIST1H2BC|HIS	1.4504973	up	histone cluster 1, H2bc | histone cluster 1,	BC105595
HIST1H2BK|H2B	1.325995	up	histone cluster 1, H2bk | H2B histone fami	AK292062|B
HIST1H3C|HIST1	1.4559077	up	histone cluster 1, H3c | histone cluster 1, I	BC127610
HIST1H4C|HIST4	1.3686852	up	histone cluster 1, H4c | histone cluster 4, I	BC130558
HP|HPR	1.4728715	up	haptoglobin | haptoglobin-related protei	AK055872
HSPG2	1.346241	down	heparan sulfate proteoglycan 2	M85289
HSPH1	1.3341	up	heat shock 105kDa/110kDa protein 1	BX648125
IBTK	1.3036625	up	inhibitor of Bruton agammaglobulinemia	DQ005633
IFI16	1.3356922	up	interferon, gamma-inducible protein 16	AF208043
IFIT2	1.3295484	up	interferon-induced protein with tetratrico	BC032839
IFNAR1	1.2957867	up	interferon (alpha, beta and omega) recep	J03171
IFRD1	1.3281156	up	interferon-related developmental regulat	BC001272
ITGA7	1.3042641	down	integrin, alpha 7	BC050280
JUP	1.3093927	down	junction plakoglobin	M23410
KIAA0195	1.3117917	down		BC042942
KIAA0515|SNOR	1.3229375	down	KIAA0515 | small nucleolar RNA, C/D box 6	BC012289
KIAA1033	1.2932938	up		BC104992
KLHL30	1.3587592	down	kelch-like 30 (Drosophila)	
KRTAP5-1	1.3639313	down	keratin associated protein 5-1	AB126070
KRTAP5-3|KRTAF	1.3152089	down	keratin associated protein 5-3 | keratin as	AB126072|A
KTN1	1.2927551	up	kinectin 1 (kinesin receptor)	AY264265
LDOC1	1.3327429	down	leucine zipper, down-regulated in cancer	AB019527
LMF2	1.35955	down	lipase maturation factor 2	BC014652
LOC401152	1.5641345	up	HCV F-transactivated protein 1	BC017399
LRP3	1.3533	down	low density lipoprotein receptor-related p	AB009462
LRP5	1.2942703	down	low density lipoprotein receptor-related p	AF077820
LTBP3|LOC10012	1.3342304	down	latent transforming growth factor beta bin	AK024477|A
LUM	1.4693182	up	lumican	BC007038
LYRM5	1.3147453	up	LYR motif containing 5	AK095164
MAN1A2	1.3279696	up	mannosidase, alpha, class 1A, member 2	BC052954
MAP1LC3B|LOC6	1.2982559	up	microtubule-associated protein 1 light ch	BC041874
MAP3K2	1.3152387	up	mitogen-activated protein kinase kinase k	AF111105
MAZ	1.2877185	down	MYC-associated zinc finger protein (purine	AB209061
MCL1|C1orf138	1.3145552	up	myeloid cell leukemia sequence 1 (BCL2-r	BC017197|A
MGC34829	1.2804462	down	Similar to hypothetical gene supported by	BC030200
MME	1.2809939	up	membrane metallo-endopeptidase	J03779
MRGPRF	1.3357364	down	MAS-related GPR, member F	BC016964
MRPL42	1.2813953	up	mitochondrial ribosomal protein L42	BC040240
MRPL47	1.3140916	up	mitochondrial ribosomal protein L47	BC032522
MYADM	1.3140979	down	myeloid-associated differentiation marke	BC095412
NAP1L1	1.2867484	up	nucleosome assembly protein 1-like 1	BC002387
NAT8L	1.7762655	down	N-acetyltransferase 8-like	BC103748
NBEAL1	1.3779292	up	neurobeachin-like 1	AY172970
NMT2	1.287753	up	N-myristoyltransferase 2	AF043325
NPHS1|PRODH2	1.2847013	down	nephrosis 1, congenital, Finnish type (nep	AF035835
NPR1	1.3369122	down	natriuretic peptide receptor A/guanylate c	BC063304
NUCB2	1.2917719	up	nucleobindin 2	AK097398
OBFC2B	1.290334	down	oligonucleotide/oligosaccharide-binding	BC006171
OBSL1	1.2971791	down	obscurin-like 1	EF063638
OMD	1.3109467	up	osteomodulin	AB000114
OR10G4|OR10G	1.2836123	down	olfactory receptor, family 10, subfamily G, member 4 |	
OR3A2|OR3A3	1.3429304	down	olfactory receptor, family 3, subfamily A, m	BC108921|B
OR4D1	1.3194876	down	olfactory receptor, family 4, subfamily D, member 1	
OR52W1	1.348034	down	olfactory receptor, family 52, subfamily W, member 1	
PA2G4	1.2947758	down	proliferation-associated 2G4, 38kDa	BC001951
PALLD	1.2804977	up	palladin, cytoskeletal associated protein	AF464873

Genesymbol	Fold change([HIGH] vs [LOW])	Regulation([HIGH] vs [LOW])	Genedescription	Genbank
PARVB	1.2815949	down	parvin, beta	AF303887
PCNP	1.2885157	up	PEST proteolytic signal containing nuclear	BC022001
PDK2	1.2969232	down	pyruvate dehydrogenase kinase, isozyme :	AK055119
PHGDH	1.3558596	down	phosphoglycerate dehydrogenase	AK093306
PICALM	1.3215449	up	phosphatidylinositol binding clathrin ass	BC048259
PIK3C2A	1.291929	up	phosphoinositide-3-kinase, class 2, alpha	BC113658
PIK3CA	1.3282412	up	phosphoinositide-3-kinase, catalytic, alpl	BC113601
PKN1	1.3285573	down	protein kinase N1	BC094766
PLD3	1.3544598	down	phospholipase D family, member 3	BC096820
PLS3	1.3176662	up	plastin 3 (T isoform)	BC056898
PLSCR3	1.3523171	down	phospholipid scramblase 3	AK124006
PNPLA2	1.3410758	down	patatin-like phospholipase domain conta	BC017280
PNPLA3	1.3764329	down	patatin-like phospholipase domain conta	BC065195
PPP2R1A	1.3007822	down	protein phosphatase 2 (formerly 2A), regul	AK290593
PRKCD	1.2924075	down	protein kinase C, delta	BC043350
PRPF40A	1.3220056	up	PRP40 pre-mRNA processing factor 40 hom	AK024810
PTMS	1.3291321	down	parathymosin	BC017025
PTPN12	1.332375	up	protein tyrosine phosphatase, non-recept	BC050008
RAB8B	1.325626	up		AB038995
RANBP3L	1.3320328	up	RAN binding protein 3-like	BC047660
RHBDD2	1.2872766	down	rhomboid domain containing 2	AF226732
RNPEPL1	1.3314698	down	arginyl aminopeptidase (aminopeptidase	BC082975
ROBO4	1.2838385	down	roundabout homolog 4, magic roundabout	AK074163
ROCK2	1.3039379	up	Rho-associated, coiled-coil containing prc	AB014519
RPE	1.2972766	up	ribulose-5-phosphate-3-epimerase	BC005148
RXRA	1.2920302	down	retinoid X receptor, alpha	BC063827
S1PR2	1.2911686	down	sphingosine-1-phosphate receptor 2	BC069598
SCCPDH	1.297631	up	saccharopine dehydrogenase (putative)	AF151807
SCFD1	1.3204099	up	sec1 family domain containing 1	AK290410
SCYL2	1.3025248	up	SCY1-like 2 (S. cerevisiae)	AK292980
SEC62	1.2813747	up		D87127
SEMA3C	1.2857058	up	sema domain, immunoglobulin domain (I	AB000220
SEPP1	1.2980919	up	selenoprotein P, plasma, 1	BC015875
SEPT7.	1.3668896	up	septin 7	AB209677
SERBP1	1.2809391	up	SERPINE1 mRNA binding protein 1	BC002488
SGCB	1.3348573	up	sarcoglycan, beta (43kDa dystrophin-asso	U31116
SLC22A17	1.2876432	down	solute carrier family 22, member 17	BC020565
SLC25A1	1.4673209	down	solute carrier family 25 (mitochondrial car	L75823
SLC29A4	1.4261646	down	solute carrier family 29 (nucleoside transp	AY485959
SLC2A4	1.5324149	down	solute carrier family 2 (facilitated glucose	BC034387
SLC39A13	1.3727272	down	solute carrier family 39 (zinc transporter),	BX647491
SLC5A3	1.4402211	up	solute carrier family 5 (inositol transporters), membe	
SLC5A6	1.2811979	down	solute carrier family 5 (sodium-dependen	AK027855
SLC7A10	1.3367475	down	solute carrier family 7, (neutral amino aci	AB037670
SLC9A3R2	1.3403714	down	solute carrier family 9 (sodium/hydrogen e	BC014513
SMCHD1	1.4377396	up	structural maintenance of chromosomes f	BC035774
SNX2	1.2851511	up	sorting nexin 2	BC003382
SNX6|RPS19	1.3398315	up	sorting nexin 6 | ribosomal protein S19	BC001798|M
SON	1.2821213	up		AF380183
SREBF1	1.4806234	down	sterol regulatory element binding transcri	BC057388
ST6GALNAC6|LO	1.3010876	down	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-g	AK057100|A
STK17B	1.4810407	up	serine/threonine kinase 17b	BC016040
STX8	1.3753562	up	syntaxin 8	AF062077
TBL1XR1	1.2845194	up	transducin (beta)-like 1 X-linked receptor	BX648935
TENC1	1.3654742	down	tensin like C1 domain containing phospha	BC150259
TM7SF2	1.2912121	down	transmembrane 7 superfamily member 2	AF023676
TMEM109	1.3384503	down	transmembrane protein 109	BC001309
TMEM115	1.3936688	down	transmembrane protein 115	BC017367
TMEM126A	1.3360947	up	transmembrane protein 126A	BC007875
TMEM185B|LOC	1.3674604	down	transmembrane protein 185B (pseudogen	BC080607|A
TMEM204	1.3196149	down	transmembrane protein 204	AY676494
TMEM88|JMJD3	1.3173466	down	transmembrane protein 88 | jumonji dom	BC057812
TMEM92	1.2842566	down	transmembrane protein 92	AY358204
TOM1L2	1.3289075	down	target of myb1-like 2 (chicken)	AY358769
TPPP3	1.3578701	down	tubulin polymerization-promoting protein	BC037798
TSKU	1.4549828	down	tsukushin	AY358317|B
TXNRD1	1.3127711	up	thioredoxin reductase 1	AK128515

Genesymbol	Fold change([HIGH] vs [LOW])	Regulation([HIGH] vs [LOW])	Genedescription	Genbank
USP53	1.3232888	up	ubiquitin specific peptidase 53	AK025301
VAMP2	1.2904371	down	vesicle-associated membrane protein 2 (s	AK021522
VCAM1	1.3510455	up	vascular cell adhesion molecule 1	BC068490
VEGFB	1.479503	down	vascular endothelial growth factor B	BC008818
VIPR1	1.2872312	down	vasoactive intestinal peptide receptor 1	AK056819
VPS13C	1.2848843	up	vacuolar protein sorting 13 homolog C (S. c	AK001243
WDR13	1.378264	down	WD repeat domain 13	AF158978
WDR57	1.3082151	up	WD repeat domain 57 (U5 snRNP specific)	EF011621
XPOT	1.2979851	up	exportin, tRNA (nuclear export receptor for	BC020569
YIPF3	1.3230605	down	Yip1 domain family, member 3	BC019297
ZC3H11A	1.3522984	up	zinc finger CCCH-type containing 11A	BC038513
ZDHHC20	1.2838407	up	zinc finger, DHHC-type containing 20	AK098818|A
ZFP36L2	1.3408856	down	zinc finger protein 36, C3H type-like 2	BC005010
ZNF385A	1.301093	down	zinc finger protein 385A	AF304052
ZNF429	1.3314047	up	zinc finger protein 429	AY269786
ZNF460	1.2801796	up	zinc finger protein 460	AY329492
ZNF706	1.2991164	up	zinc finger protein 706	AF275802|B
ZNF91	1.3351623	up	zinc finger protein 91	AK291256

Table B: eQTL analysis results. (Chapter 4)

GENE	CHR	SNP	UNADJ	GC	BONF	HOLM	SIDAK_SS	SIDAK_SD	FDR_BH	FDR_BY	MATCH CHR	CODING
HECTD3	15	rs1158683	7.982e-009	1.201e-008	0.002447	0.002447	0.002444	0.002444	0.002447	0.03233	N	slco3a1/ptprd
UGCGL1	2	rs7563853	3.324e-008	4.351e-008	0.01019	0.01019	0.01014	0.01014	0.01019	0.1346	Y	
AZI2	9	rs7856797	3.009e-008	3.853e-008	0.009225	0.009225	0.009183	0.009183	0.009225	0.1219	N	ptprd
CP	3	rs10511083	7.82e-008	9.761e-008	0.02398	0.02398	0.02369	0.02369	0.02398	0.3167	Y	cadm2
GZMK	4	rs6835588	2.234e-008	2.666e-008	0.006849	0.006849	0.006826	0.006826	0.0026	0.03434	N	fam190a
LY6G5B	6	rs9398869	3.348e-008	4.701e-008	0.01026	0.01026	0.01021	0.01021	0.01026	0.1356	Y	ptprk
AMD1	19	rs2287851	2.906e-008	3.822e-008	0.008912	0.008912	0.008872	0.008872	0.008912	0.1177	N	unc13a
FAM92A1	17	rs1345451	3.89e-009	5.602e-009	0.001193	0.001193	0.001192	0.001192	0.001193	0.01576	N	ccdc46
	17	rs997651	1.829e-008	2.613e-008	0.005606	0.005606	0.005591	0.005591	0.002771	0.0366	N	ccdc46
	17	rs3744318	2.711e-008	3.831e-008	0.008312	0.008312	0.008277	0.008277	0.002771	0.0366	N	ccdc46
SPAG11B	18	rs470563	2.069e-008	2.678e-008	0.006345	0.006345	0.006325	0.006325	0.006345	0.08382	N	znf236
FAM69B	2	rs6546473	5.188e-008	6.531e-008	0.01591	0.01591	0.01578	0.01578	0.01591	0.2101	N	antxr1
EEA1	22	rs861826	2.253e-008	3.117e-008	0.006908	0.006908	0.006885	0.006885	0.006908	0.09126	N	ppil2
DICER1	12	rs7956904	2.646e-010	5.068e-010	8.112e-005	8.112e-005	8.111e-005	8.111e-005	8.112e-005	0.001072	N	kcna6
TOM1L2	17	rs9303380	2.272e-009	3.569e-009	0.0006966	0.0006966	0.0006963	0.0006963	0.0006966	0.009202	Y	n/a
	11	rs4910365	9.121e-009	1.41e-008	0.002797	0.002797	0.002793	0.002793	0.001398	0.01847	N	galntl4
NUFIP2	12	rs7956904	1.669e-008	2.504e-008	0.005116	0.005116	0.005103	0.005103	0.005116	0.06759	N	kcna6

GENE, eQTL; CHR, Chromosome; SNP, SNP identifier; UNADJ, Unadjusted asymptotic significance value; GC, Genomic control adjusted significance value. This is based on a simple estimation of the inflation factor based on median chi-square statistic. These values do not control for multiple testing therefore; BONF, Bonferroni adjusted significance value; HOLM, Holm step-down adjusted significance value; SIDAK_SS, Sidak single-step adjusted significance value; SIDAK_SD, Sidak step-down adjusted significance value; FDR_BH, Benjamini & Hochberg (1995) step-up FDR control; BY, Benjamini & Yekutieli (2001) step-up FDR control

Figure C: rs861826 in LD with splice/non-synonymous coding SNP. PPIL2-EEA1 (Chapter 4)

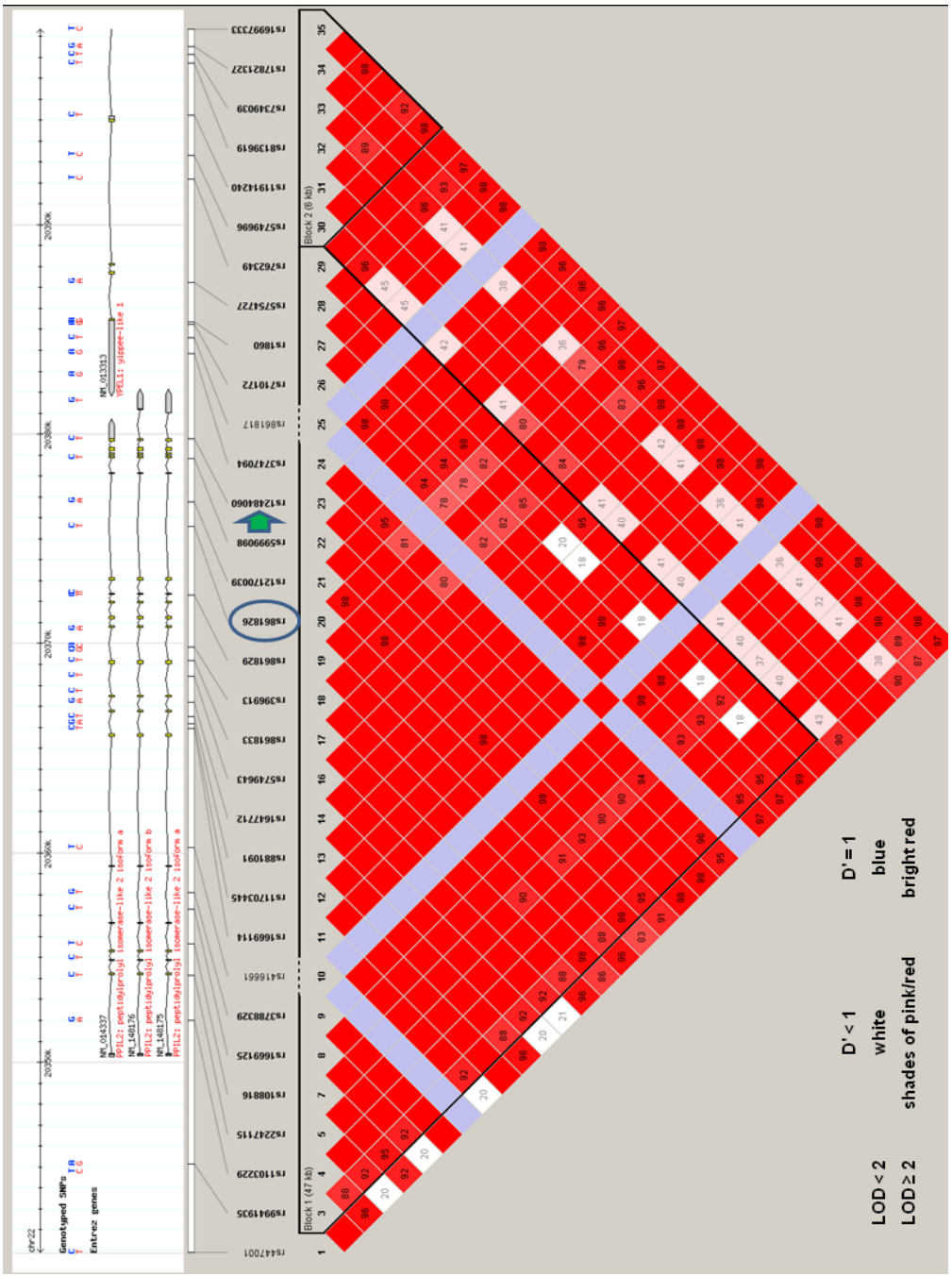

Figure D: SREBP-1a mRNA expression (Chapter 4)